Characterization of PII and truncated PII transgenic *Arabidopsis thaliana*

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Abstract

PII is an important regulatory protein controlling nitrogen metabolism in bacteria. Recent researches show that PII is not limited to prokaryotes but is present also in some eukaryotes such as red algae, castor bean, alfafa and *Arabidopsis thaliana*. The PII homologs of *Arabidopsis thaliana* exhibit high overall homology (50%) with the conserved signature domains of the *Escherichia coli* counterpart. Moreover, PII mRNA of *Arabidopsis thaliana* is induced by light and carbon metabolites and is repressed by organic nitrogen such as amino acids. Based on these initial findings, PII is suggested to take part in the complex signal transduction pathways involved in sensing the status of carbon and nitrogen in higher plants, which still remain unexplored.

Transgenic strains of *Arabidopsis thaliana* overproducing normal (PII24-1T4 & PII1-13T3) or truncated PII (TPII3-6T2 & TPII6-9T2) proteins were constructed previously. Using these transgenic lines, the effects of PII on nitrogen assimilation and hence plant growth and development were studied. Analysis of the general growth pattern revealed impaired growth in the case of the truncated PII transgenic lines. Physiological tests were also performed to measure plant growth using the following parameters: fresh weight, root length, chlorophyll content and seed nitrogen content. Under different nitrogen status, PII and truncated PII overexpressing transgenic lines showed significant differences in the root length, seedling chlorophyll content and seed nitrogen content, compared to the untransformed Col-0. Such deflects in growth and development may result from altered nitrogen sensing efficiency in these transgenic lines.

Furthermore, Northern blot analysis was performed to test if overexpressing normal PII and truncated PII will directly or indirectly interact with other nitrogen assimilatory

genes, including *NIA1*, *NIA2*, *GSL1*, *GSR2*, *ASN1*, *ASN2* and *ASN3*. The results indicated that both the PII and truncated PII transgenic lines showed reduced levels of *NIA1*, *NIA2*, *ASN2* and *ASN3*, compared to Col-0. In contrast, in the truncated PII transgenic lines, a slightly elevated level of *GSL1* steady-state mRNA was observed. A model was proposed to explain the above observations and to summarize the possible effects of PII on nitrogen metabolism in higher plants.

摘要

PII 是細菌氮代謝過程中一個重要的調控蛋白。除細菌外，最近的研究亦相繼在一些真核生物如紅藻、蓖麻、苜蓿及擬南芥菜內發現 PII 蛋白基因。其中擬南芥菜和蓖麻的 PII 蛋白與大腸桿菌 PII 蛋白有相當高的整體同源性(達百分之五十)。實驗結果顯示光照或碳代謝物提升擬南芥菜 PII 蛋白基因的表達水平；而黑暗或有機氨基酸則會降低它的表達水平。基於此等發現，PII 可能是一個複雜的碳和氮感應訊號傳遞系統的一部份，而目前尚未有相關的研究報告。

從前的研究已成功製造出超量表達 PII (PII24-1T4 & PII1-13T3) 或截短 PII(TPII3-6T2 & TPII6-9T2) 的轉基因擬南芥菜。利用這些轉基因擬南芥菜，是項研究嘗試分析 PII 蛋白在氮代謝中所起之作用及其對整體生長與發育之影響。從普通生長狀態的觀察發現，截短 PII 轉基因品系的生長受到損害。進一步的生理實驗檢測了下列植物生長參照性狀：淨重、根長、葉綠素含量和種子氮含量。在不同氮供應情況下，PII 及截短 PII 轉基因品系與非轉化親本 Col-0 在根長、幼苗葉綠素含量以及種子氮含量均有明顯差異。這些生長及發育上的偏差可能是基於此等轉基因品系內氮感應效率之改變。

除此之外，爲了探究超量表達 PII 及截短 PII 會否直接或間接地影響其他氮同化相關基因，是項研究應用北方印跡法測試了 *NIA1*, *NIA2*, *GSL1*, *GSR2*, *ASN1*, *ASN2* and *ASN3* 等基因。結果顯示 PII 及截短 PII 轉基因品系內的 *NIA1*, *NIA2*, *ASN2* 以及 *ASN3* 的穩定狀態 mRNA 均較 Col-0 爲少。相反地，在截短 PII 轉基因品系內的 *GSL1* 穩定狀態 mRNA 則較 Col-0 爲高。最後，此項研究提出了一個模型來解釋以上結果並總結 PII 在高等植物氮代謝中的可能作用。

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Abbreviations

Bp	Base pair
BSA	Bovine serum albumin
C:I	Chloroform and Isoamyl alcohol (24:1) solution
Col-0	Columbia zero
DNA	Deoxyribonucleic acid
dNTP	Deoxyriboucleoside triphosphate
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DTT	Dithiothreitol
EDTA	Ethylenediamine-tetraacetic acid
g	Gram
Glu	Glutamine
mRNA	Messenger ribonucleic acid
µl	Microliter
µM	Micromolar
ml	Milliliter
mM	Millimolar
ng	Nanogram
O.D.	Optical density
Phe	Phenylalanine
P:C:I	Phenol: chloroform: Isoamyl alcohol (25:24:1) solution
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
Ser	Serine
Tris base	Tris (hydroxymethyl) aminomethane hydrochloric acid
TE	Tris-EDTA
Tyr	Tyrosine

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1. Literature Review

In the past 35 years, the rate of grain production has doubly increased to meet the requirement of the 5.8 billion global human population (Tilman, 1999). Such an impressive accomplishment was associated with a 6.87-fold increase in nitrogen fertilizer applications, a 3.48-fold increase in phosphorus fertilizer applications, an 1.68-fold increase in the amount of irrigated cropland and an 1.1-fold increase in cultivation on land (Tilman, 1999). To cope with the projected peak of global human population in 2030 (Kawashima *et al.*, 1997), agricultural production should be increased accordingly. However, spectacular growth in grain production in the past 35 years is expected to slow down because further additions of the nitrogen, phosphorus and irrigation are no longer effective in increasing the yield. As a result, the amount of land dedicated to agriculture may have to increase disproportionately to fulfill the needed gain in global food production. Such increases in cultivated land and applications of nitrogen and phosphorus fertilizers, together with irrigation practice would result in severe environmental damages and may even lead to ecosystem destruction (Tilman, 1999). To cope with such a challenge, advances in biotechnology may bring possible solutions.

In particular, basic studies of nitrogen metabolism at the molecular level in plants are of utmost importance. Nitrogen is one of the essential elements for all kinds of living

organisms included bacteria, plants and animals. Even though there is abundant of nitrogen (78% v/v) in air, atmospheric nitrogen cannot be used directly by most of the organisms. In plants, inorganic nitrogen must be converted into ammonia (by nitrogen fixation in legumes or nitrate reduction in other plants) first before assimilated into other nitrogen-containing organic compounds.

1.1 GS-GOGAT cycle in plants and bacteria

In both bacteria and plants, ammonia is mainly assimilated via the "GS/GOGAT cycle". In this cycle, while glutamine oxoglutarate aminotransferase (GOGAT) catalyzes the transfer of the amide group from glutamine to the keto group of α-ketoglutarate to produce two molecules of glutamate, glutamine synthetase (GS) on the other hand catalyzes the synthesis of glutamine from ammonia and glutamate. Together, GS and GOGAT catalyze the net synthesis of glutamate from α-ketoglutarate, ammonia and NADPH at the cost of ATP hydrolysis (Figure 1.1). This cycle continues as long as ammonia and the required substrates are adequate. Therefore, GS is an important enzyme in regulating the overall nitrogen assimilation by acting as the entry point into the GS-GOGAT cycle.

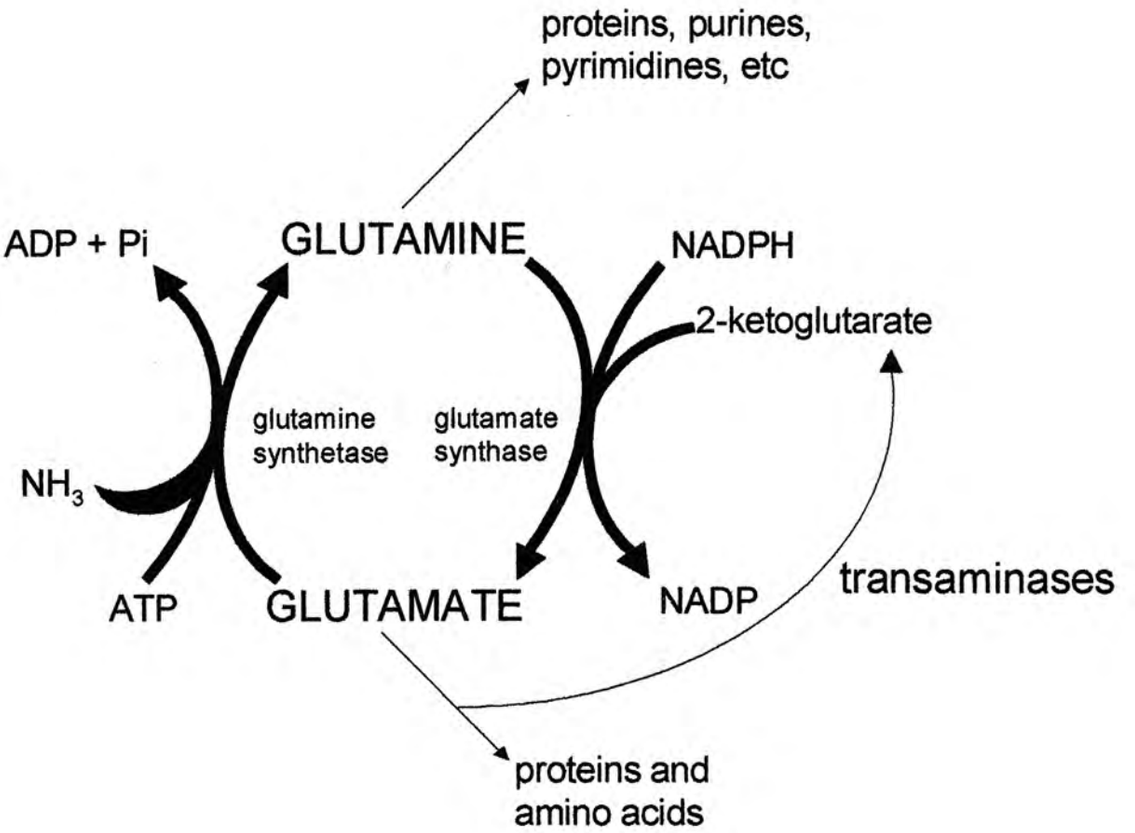


Figure 1.1: GS/GOGAT cycle in plants and bacteria (Reitzer & Magasanik, 1987).

1.2 Roles of PII in the regulation of glutamine synthetase in *E. coli*

In growth medium containing high ammonium (>1mM), *E. coli* synthesizes glutamate by reductive amination of α-ketoglutarate (an important intermediate in carbon metabolism), a reaction catalyzed by the NADP-linked glutamate dehydrogenase (GDH). Under this circumstance, about 85% of the cellular nitrogen is derived from the amino nitrogen of glutamate synthesized by GDH and only 15% from the amide nitrogen of glutamine synthesized by GS.

However, when the ammonia concentration in the medium is below 1mM, GDH activities drop drastically due to its high K_m to ammonium. The GS-GOGAT will then provide the main pathway for primary nitrogen assimilation.

1.2.1 Regulation of GS in *E. coli*

In *E. coli*, GS is regulated at three levels: transcriptional regulation, post-translational modification and cumulative feedback inhibition of the GS enzyme. Both transcriptional and post-translational regulations use a common sensing mechanism in which the PII protein is involved (Kamberov *et al.*, 1995). PII is a homotrimeric protein that has a barrel-like core with recognition loops (the T-loops) protruding from the top of the molecules. Each monomer consists of a central core with a B-

loop, T-loop and a C-loop (Carr *et al.*, 1996; Cheah *et al.*, 1994). Previous studies suggested that the T-loop and the cleft formed between the T-, B- and C-loops are the sites of important regulatory interactions, including those for small-molecule effectors and/or protein receptors (Cheah *et al.*, 1994; Jiang *et al.*, 1997).

1.2.2 Transcriptional regulation

1.2.2.1 The glnALG operon

The *glnALG* operon, located at 87 minute on the *E. coli* genome, is responsible for synthesizing GS (Backman *et al.*, 1981, Magasanik, 1988). It contains *glnA*, the structural gene for glutamine synthetase, as well as *glnL* and *glnG* encoding the nitrogen regulator I (NRI) and nitrogen regulator II (NRII), respectively (Figure 1.2, Table 1.1). The two-component system consisting of a modulator (NRII) and a response regulator (NRI) (Magasanik, 1988) are responsible for the regulation of transcription of the *glnALG* operon (Reitzer & Magasanik, 1983) as well as other nitrogen regulated genes (Magasanik, 1982) (Magasanik, 1988; Tyler, 1978). The operon is endowed with three promoters, *glnAp1*, *glnAp2* and *glnLp*. Promoters *glnAp1* and *glnAp2* are located upstream of *glnA* while the promoter *glnLp* is located between *glnA* and the *glnLG* portion of the operon. The *glnLp* region arrests approximately 80% of the transcripts that have passed

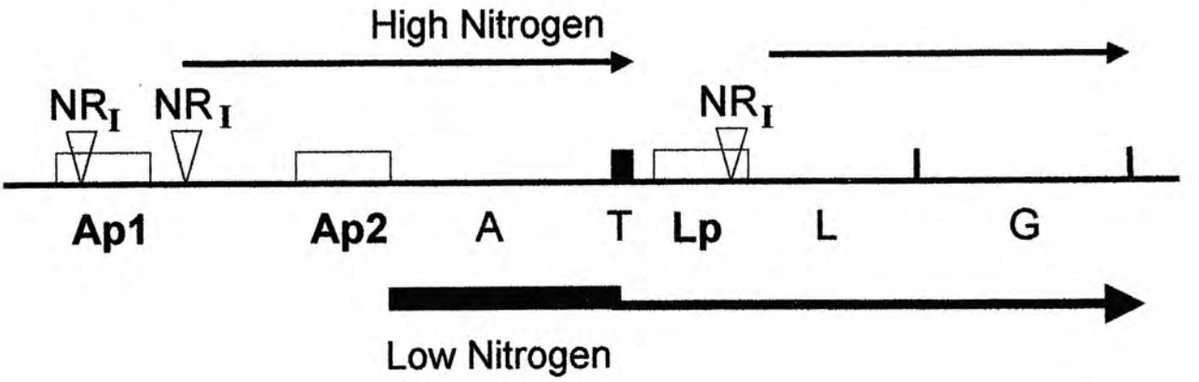


Figure 1.2: Transcription of the *glnALG* operon in *E. coli.* The bacterial cells were grown when ammonia is in excess (high N) or limiting (Low N), □ , promoters Ap1, Ap2 and Lp; ▽, NRI-binding sites; ▮, terminator; ⟶ , direction of transcripts (Magasanik, 1988).

Table 1.1: Genes involved in ammonium assimilation in *E. coli.*

Gene	Glossary of *gln* genes and proteins[a]	References
glnA	GS	(Miranda-Rios *et al.*, 1987)
glnB	PII	(Heung & Sue, 1987)
glnD	UTase/UR	(van Heeswijk *et al.*, 1993)
glnE	ATase	(van Heeswijk *et al.*, 1993)
glnF	σ^{54}	(Sasse-Dwight & Gralla, 1990)
glnG	NRI	(McFarland *et al.*, 1981) (Miranda-Rios *et al.*, 1987)
glnL	NRII	(McFarland *et al.*, 1981) (Miranda-Rios *et al.*, 1987)
glnK	PII	(van Heeswijk *et al.*, 1996)

[a] Abbreviations: GS, glutamine synthetase; UTase/UR, uridylyltransferase/uridylyl-removing enzyme; ATase, adenylyltransferase; NRI, nitrogen regulator; NRII, nitrogen regulator II.

through *glnA* (Magasanik, 1996).

1.2.2.2 Intracellular signal through PII and uridylyltransferase/uridylyl-removing enzyme (UTase-UR)

Regulation of GS is complex and the proper operation of the system requires many components including PII, UTase-UR, NRI, NRII, σ^{54} and GS itself (Table 1.1). This complex regulatory system responds immediately to the change of nitrogen status in the environment and such high sensitivity enables the cells to rapidly and profoundly adjust the rate of GS synthesis to meet the nitrogen demand.

When nitrogen supply is abundant, the *glnA* and *glnLG* transcription are initiated by a common σ^{70}-RNA polymerase at promoter *glnAp1* and *glnLp*, respectively (Hunt & Magasanik, 1985). The transcription initiated at *glnAp1* and *glnLp* are repressed by the *glnG* product, NRI at the binding sites for NRI overlap –35 region at *glnAp1* and the –10 region at *glnLp*, respectively.

When ammonia is in excess, NRI partially represses *glnA* transcription as well as *glnG* (its own gene) and *glnL* (encoding for NRII) synthesis by partially blocking the initiation of *glnALG* transcription at the promoter *glnAp1* and *glnLp*. Approximately five molecules of NRI are present in a cell under such condition. The σ^{54}-RNA polymerase is bound to the *glnAp2* promoter as part of a closed complex and no

transcription is initiated at this promoter (Sasse-Dwight & Gralla, 1988). Ammonia deprivation results in phosphorylation of NRI. Phosporylated NRI binds to the two strong binding sites overlapping the *glnAp1* promoter that are distal to the σ^{54}-dependent *glnAp2* promoter. These two binding sites are the prokaryotic equivalents of eukaryotic enhancers. The binding of phosphorylated NRI to these sites catalyzes the transformation of the closed to the open complex and consequently activates the transcription initiation at *glnAp2* (Ninfa & Magasanik, 1986; Sasse-Dwight & Gralla, 1988). This results in a large increase of GS (product of *glnA*) and NRI (product of *glnG*). The increased intracellular concentration of NRI causes complete arrest of transcription initiation at the *glnAp1* and *glnLp* promoters. Meanwhile, *glnAp2* serves as the sole promoter of the *glnALG* operon. A cell deprived of nitrogen contains approximately 70 molecules of NRI and such high intracellular concentration of NRI further activates other nitrogen-regulated promoters. Therefore, the first response of the cell to ammonia deficiency is increased synthesis of glutamine synthetase which allows the cell to utilize ammonia present in low concentration in the medium, followed by increased synthesis of enzymes and permeases that allow it to use alternative sources of nitrogen (Magasanik, 1988).

1.2.2.3 NRI/NRII as two-component system

As described above, binding of phosphorylated NRI to the specific promoter results in an elevated transcription of *glnA* at the promoter *glnAp2* whereas unphosphorylated NRI represses the transcription of *glnA* at the promoter *glnAp1* and *glnLp*.

NRI itself is regulated by NRII, the gene product of *glnL*. Together, these two molecules form a two-component system in which a modulator (NRII) phosphorylates a response regulator (NRI) in response to the specific signals from PII.

The NRI/NRII two-component system works jointly with PII and UTase-UR and transduces the intracellular signal of the nitrogen status reflected from the glutamine to α-ketoglutarate ratio. GS in turn may act as an ammonia sensor that regulates the glutamine to α-ketoglutarate ratio. The balance between nitrogen and carbon metabolism as reflected by the intracellular ratio of glutamine and α-ketoglutarate appears to be the critical signal (Jiang *et al.*, 1998; Stadtman & Chock, 1978).

A high glutamine to α-ketoglutarate ratio (indicating nitrogen abundancy) stimulates the deuridylylation of PII-UMP to PII by the enzyme UTase-UR. The presence of unmodified PII activates NRII to catalyze the removal of the phosphate from

phosphorylated NRI. This results in the cessation of *glnA* transcription at promoters *glnAp1* and *glnLp* (Reitzer and Magasanik, 1987, Reitzer and Magasanik, 1986). Conversely, a low glutamine to α-ketoglutarate ratio (indicating nitrogen deficiency) stimulates the uridylylation of PII to PII-UMP by the same enzyme, UTase-UR. Removal of PII allows the NRII to act as a kinase to phosphorylate NRI which in turn activates the transcription at the promoter *glnAp2*. The regulation through PII is demonstrated in Figure 1.3.

1.2.3 Post-translational regulation by adenylylation and deadenylylation

Apart from transcriptional control, GS is also subjected to post-translational regulation. Bacterial GS consists of 12 identical subunits and each subunit can be separately adenylylated or deadenylated (Kingdon *et al.*, 1967, Anderson *et al.*, 1970). When GS is adenylylated by adenylyltransferase (ATase), it will be converted into a less active form. When the adenyl group is removed by the same enzyme, GS is reverted back to the active form. Thus, the overall GS activity is finely adjusted and controlled by the average number of adenylylation subunits per molecules that can vary from 0 to 12 (Kingdon *et al.*, 1967).

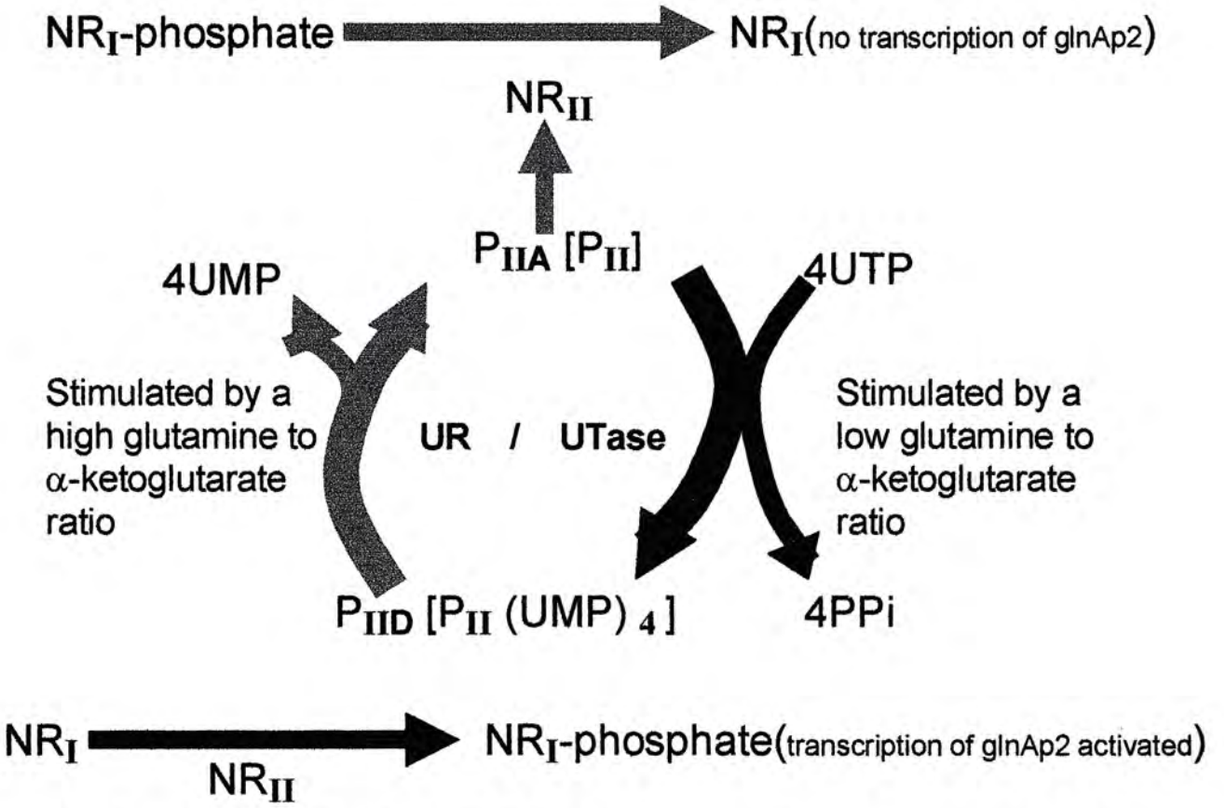


Figure 1.3: Covalent modification of NRI, the regulator of *glnA* transcription under different glutamine/α-ketoglutarate ratio (Reitzer & Magasanik, 1987).

1.2.3.1 Role of PII in adenylylation/deadenylylation

The complex regulatory system that control GS activity consists essentially of two enzyme-catalyzed interconnected interconversion systems, including the PII regulatory protein-uridylylation system and GS adenylylation system (Figure 1.4). A high glutamine to α-ketoglutarate ratio triggers the activation of UTase-UR which catalyzes the deuridylylation of PII-UMP to PII. The presence of PII stimulates adenylyltransferase to catalyze the adenylylation of GS. The resulting adenylylated GS is less active with altered pH optimum and co-factor dependent (Adler *et al.*, 1975).

A similar cascade leads to the activation of GS enzyme. It is initiated by a low glutamine to α-ketoglutarate ratio. UTase-UR catalyzes the covalent attachment of UMP to PII. The uridylylated PII interacts with adenylyltransferase to activate the deadenylylation of GS and converts it back to the more active Mg^{2+}-dependent form. Through these complex regulatory systems, GS enzyme activity is finely modulated by various metabolites includes UTP, ATP, 2-ketoglutarate, Pi, glutamine and probably other compounds yet to be identified (Adler *et al.*, 1975).

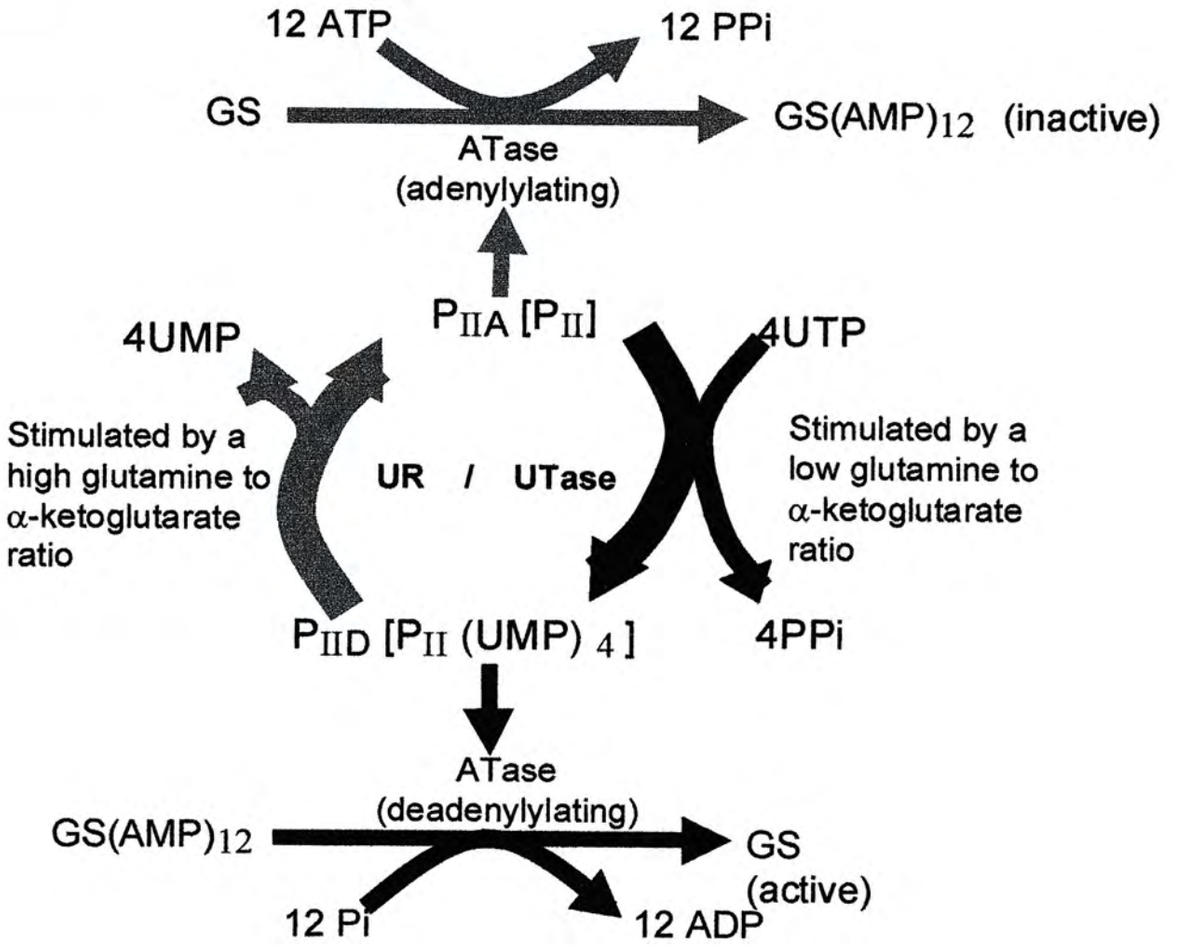


Figure 1.4: Covalent modification of GS enzyme by the action of PII and ATase under different glutamine/α-ketoglutarate ratio (Reitzer & Magasanik, 1987).

1.2.4 Cumulative Feedback Inhibition

GS is responsible for two physiological functions: 1) formation of glutamine for protein synthesis; and 2) assimilation of ammonia. Cumulative feedback inhibition only affects the adenylylated GS, the modified form when ammonia is in excess (Stadtman & Ginsburg, 1974).

When bacterial cells are grown in a nitrogen-limited medium, unadenylylated GS functions primarily to assimilate ammonia, and is not susceptible to feedback inhibition. However, in ammonia-containing medium, the adenylylated GS functions primarily in the formation of glutamine for the synthesis of protein and other nitrogenous compounds. The enzyme is not needed for the ammonia assimilation. This adenylylated enzyme activity is inhibited by the products of glutamine metabolism include CTP, ATP, glucosamine-6-P, histidine, tryptophan, carbamyl-P alanine, glycine and serine (Rhee *et al.*, 1985, Reitzer and Magasanik, 1987).

1.3 PII in other bacteria

Besides *E.coli*, PII protein also exists in a wide range of other enteric bacteria included both Gram-negative and high-GC-Gram-positive bacteria (Pesole *et al.*, 1995). Extensive studies were done on these bacteria including *E. coli*, *Streptomyces coelicolo*r, *Rhizobium leguminosarum*, *Rhodobacter capsulatus*, *Rhizobium meliloti*,

Azospirillum brasilense, *Klebsiella aerogenes*, Cyanobacterium *Synechococcus sp. and Nostoc punctiforme*, etc.

Despite the broad existence of genes encoding PII-like proteins in various organisms, its functions have been defined in only a few bacteria. PII is best known as a signaling protein (see above) in the regulation of GS in *E. coli* and *K. pneumoniae*. Yet, many studies suggested that PII may also provide other functions that are related to nitrogen metabolism (Table 1.2).

Studies in *Rhizobium leguminosarum* suggested that PII protein is necessary for the expression of genes required for nitrate uptake and/or reduction in addition to the regulation of GS,(Amar *et al.*, 1994).

Besides, it is also proposed that the *Rhizobium meliloti* PII protein affect alfalfa nodule development through the control of expression or activity of a bacteroid ammonium transporter (Arcondeguy *et al.*, 1997).

In *Azotobacter vinelandi*, PII protein influences the regulation of the *nif* gene expression in response to the ammonium fluxes. Similar function was proposed in *Klebsiella pneumoniae* that a PII-like protein (*GlnK*) is responsible for the NifL-dependent nitrogen control of *nif* gene expression according to the nitrogen supply (He *et al.*, 1998).

Moreover, PII in diazotrophic bacterium *Azospirillum brasilense* is found to be

Table 1.2: Functions of the PII gene and its homologs in different organisms

Organisms	Genes	**Functions**	**References**
Arabidopsis thaliana	*GLB1*	▪ Possibly involved in nitrogen sensing.	Hsieh *et al.*, 1998
Azorhizobium caulinodans	*GlnB*	▪ Involved in the regulation of nitrogen fixation. ▪ Together with *glnK*, it is required for complete GS deadenylylation.	(Michel-Reydellet & Kaminski, 1999)
Azorhizobium caulinodans	*GlnK*	▪ Involved in the regulation of nitrogen fixation. ▪ Together with *glnB*, it is required for the complete GS deadenylylation.	(Michel-Reydellet & Kaminski, 1999)
Azospirillum brasilense	*GlnB*	▪ Regulation of nitrogen fixation by modulating NifA activity. ▪ Controlling swarming properties of the cells. ▪ Not essential for the adenylylation of GS.	(de Zamaroczy *et al.*, 1993) (de Zamaroczy *et al.*, 1996)
Azospirillum brasilense	*GlnZ*	▪ Functions unclear, it may be involved in an alternative intracellular nitrogen signaling. ▪ Not required for the nitrogen control of GS activity.	(de Zamaroczy *et al.*, 1996) (de Zamaroczy, 1998)
E. coli	*GlnB*	▪ Mediate the modulation of glutamine synthetase adenylylation and deadenylylation. ▪ Regulate the expression of the glutamine sythetase gene *glnA*.	(Brown *et al.*, 1971)
E. coli	*GlnK*	▪ Together with *glnB* product to form heterotrimers that fine-tune the nitrogen signal cascade. ▪ Regulate the expression of the level of phosphorylated NRI during conditions of severe nitrogen starvation and hence involved in regulation of certain *Ntr* genes.	(van Heeswijk *et al.*, 2000) (Atkinson & Ninfa, 1998)

Klebasiella pneumoniae	*GlnK*	▪ Relief of NifL inhibition on the NifA activity that regulate the nitrogen fixation (*nif*) genes in response to nitrogen status.	(He *et al.*, 1998) (Jack *et al.*, 1999)
Synechococcus sp. PCC 7942	*GlnB*	▪ Involved in global nitrogen control. ▪ Tight coordination of the carbon and nitrogen assimilation. ▪ Regulation of nitrate and nitrite uptake.	(Forchhammer & de Marsac, 1994) (Forchhammer & de Marsac, 1995) (Lee *et al.*, 1998)
Synechococcus sp. PCC 6301	*GlnB*	▪ Possibly a central integrator in the interaction between photosynthesis and nitrogen assimilation.	(Tsinoremas *et al.*, 1991)
Synechocystic sp. PCC 6803	*GlnB*	▪ Regulation of the inorganic carbon uptake. ▪ Regulation of nitrate uptake.	(Hisbergues *et al.*, 1999)
Rhizobium legminosarum	*GlnB*	▪ Positive effector of the expression of nitrate utilization genes (*nas* genes).	(Amar *et al.*, 1994)
Rhdobacter capsulatus	*GlnB*	▪ Mediate the modulation of glutamine synthetase adenylylation and deadenylylation. ▪ A negative regulator of *nif* gene transcription.	(Kranz & Foster-Hartnett, 1990) (Kranz & Haselkorn, 1985)
Streptomyces coelicolor A3(2) (find journal about PII)	*GlnB*	▪ Mediate the modulation of glutamine synthetase adenylylation and deadenylylation.	(Fink *et al.*, 1999)

required to modulate the activity of NifA which is a transcriptional activator for the nitrogen fixation genes (Arsene *et al.*, 1996).

In cyanobacteria, no NRI/NRII homologs are identified and there is no evidence to indicate the existence of an adenylylation control of GS (Tandeau de Marsac & Houmard, 1993).

However, the *glnB* gene product has been identified in various cyanobacteria include the *Synechococcus* PCC 7942 (Tsinoremas *et al.*, 1991), *Synechococcus* PCC 6803 (Garcia-Dominguez & Florencio, 1997), *Anabaena* PCC 7120 (Gonzalez, L., Phalip,V. and Zhang,C.C., unpublished), *Nostoc punctiforme* ATCC 29133 (Hanson *et al.*, 1998b) and *Synechococcus* PCC 6301 (Harrison *et al.*, 1990).

PII in cyanobacteria was characterized and subjected to functional analysis. Functioning as part of the complex signal transduction network in the global nitrogen control, PII protein of both *Synechococcus* PCC 7942 and PCC 6803 may involve in mediating the tight coordination between carbon and nitrogen assimilation, (Forchhammer & de Marsac, 1995; Hisbergues *et al.*, 1999) and the regulation of nitrate and nitrite uptake, depending on the intracellular N/C balance (Lee *et al.*, 2000, Lee *et al.*, 1998) as well as the inorganic carbon uptake (Hisbergues *et al.*, 1999).

The *glnB* gene and its gene product PII in cyanobacteria are different from that of *E.*

coli with respect to the gene expression regulation and protein modification. Detailed comparisons were done and shown in Table 1.2 and 1.3.

In contrast to the constitutively expressed *glnB* gene of *E. coli* (van Heeswijk *et al.*, 1993), expression of Synechocystic PCC 6803 *glnB* was specifically regulated by carbon availability, nitrogen availability, light-dark transitions and photosynthetic inhibitors (Garcia-Dominguez & Florencio, 1997).

Similarly, *glnB* expression in other bacteria such as *B. japonicum*, *A. brasilense* and *R. capsulatus* are also regulated at the transcriptional level in response to nitrogen availability (de Zamaroczy *et al.*, 1993, Foster-Hartnett and Kranz, 1994, Martin *et al.*, 1989b).

Besides transcriptional regulation, PII is also subjected to post-translationally modification in cyanobacteria. However, contrasting to the uridylylation at the conserved tyrosine residue in *E. coli* and other enteric bacteria, cyanobacterial PII is modified by phosphorylation on a serine residue (Forchhammer and de Marsac, 1994, Brown *et al.*, 1971, Magasanik, 1993).

1.4 PII in other higher organisms

Despite the extensive studies on PII protein and its regulation of nitrogen assimilation in bacteria, it is until recently that *glnB* gene was discovered in

Table 1.3: Regulatory modification of the PII protein and its homologs in different organisms

Organisms	Gene	Posttranslational modification	Remarks	References
Arabidopsis	*GLB1*	Unknown, but it possesses a phosphorylation site at ser49	▪ Induced by sucrose and light. ▪ Repressed by amino acids.	(Hsieh *et al.*, 1998)
Azorhizobium caulinodans	*GlnB*		▪ Constitutively expressed in any nitrogen status.	(Michel-Reydellet & Kaminski, 1999) (Michel-Reydellet *et al.*, 1997)
Azorhizobium caulinodans	*GlnK*		▪ Induced by ammonia	(Michel-Reydellet & Kaminski, 1999)
Azospirillum brasilense	*GlnB*	Uridylylation	▪ Transcription using a σ^{54}-like promoter. ▪ Regulated by NRI according to the nitrogen status.	(de Zamaroczy *et al.*, 1993)
Azospirillum brasilense	*GlnZ*	Uridylylation	▪ Transcription using σ^{54}-like promoter. ▪ Activated by NRI. ▪ Expressed even under conditions of nitrogen excess.	(de Zamaroczy *et al.*, 1996)
Bradyrhizobium japonicum	*GlnB*		▪ Induced by NRI.	(Martin *et al.*, 1989a)
E. coli	*GlnB*	Uridylylation at Tyr51	▪ Constitutively expressed in any nitrogen status.	(Brown *et al.*, 1971) (Jaggi *et al.*, 1996)
E. coli	*GlnK*	Uridylylation at Tyr51	▪ Regulated with respect to the intracellular nitrogen status	(van Heeswijk *et al.*, 1996) (Jiang *et al.*, 1997) (Atkinson & Ninfa, 1999) (Jaggi *et al.*, 1996) (van Heeswijk *et al.*, 1993) (Atkinson & Ninfa, 1998)

Klebsiella pneumoniae	*GlnB*	Uridylylation		(Holtel & Merrick, 1988)
Klebsiella pneumoniae	*GlnK*	Uridylylation	▪ Required NRI.	(He *et al.*, 1998) (van Heeswijk *et al.*, 1996) (Jack *et al.*, 1999)
Nostoc punctiforme strain ATCC 29133	*GlnB*	Phosphorylated *in vitro*		(Hanson *et al.*, 1998a)
Rhizobium leguminosarum	*GlnB*		▪ GlnB gene was located upstream of glnA gene and together to form the glnBA operon. ▪ It is regulated by NRI/NRII according to the nitrogen availability.	(Amar *et al.*, 1994)
Rhodobacter capsulatus	*GlnB*		▪ It is regulated by NRI.	(Foster-Hartnett & Kranz, 1994)
Synechococcus sp. 6301	*GlnB*	Phosphorylation at ser49	▪ NtcA regulates PII at the transcriptional level.	(Harrison *et al.*, 1990) (Tsinoremas *et al.*, 1991)
Synechococcus sp. PCC 7942	*GlnB*	Phosphorylation at ser49	▪ NtcA regulates PII at the transcriptional level.	(Forchhammer & Hedler, 1997) (Forchhammer & de Marsac, 1994) (Forchhammer & de Marsac, 1995) (Lee *et al.*, 1999)

eukaryotes such as algae and higher plants. Studies on the eukaryotic PII and the related regulation of nitrogen assimilation have just begun.

The first *glnB* gene identified in eukaryotes is from the red algae *Porphyra purpurea* (Reith & Munholland, 1993). Recently, *glnB* genes in higher plants were identified in *Medicago sativa* (alfalfa) (Garcia-Ibilcieta, D. and Sengupta-Gopalan, C. unpublished), *Ricinus communis* (castor bean) (Hsieh *et al.*, 1998) and *Arabidopsis thaliana* (Hsieh *et al.*, 1998). The *GLB1* cDNA (encoding for PII protein) clone of castor bean was obtained by mass sequencing of a λZAPII library containing cDNAs of developing endosperm and embryos of castor bean. Based on sequence homology to the bacterial PII (Hsieh *et al.*, 1998). Using this clone, the *GLB1* cDNA of *Arabidopsis thaliana* was obtained by screening a cDNA library, using the castor bean *GLB1* gene as a heterologous probe (Hsieh *et al.*, 1998). Therefore, the *glnB* gene seems to be conserved and plays crucial function in various domains of life. Possible functions and posttranslational modification of PII protein and its homologs in different organisms were summarized in Table 1.2 and Table 1.3.

1.5 PII protein is conserved in enteric bacteria, cyanobacteria, Archaea, algae and higher plants

Representative bacterial and archaea PII amino acid sequences were aligned with

plant PII sequences. The overall homology between the plant PII and the *E. coli* PII is 50% (Hsieh *et al.*, 1998). The deduced amino acid sequences of the plant PII also share high overall identities to other microbial *glnB* genes, including *Synechosystis* sp. PCC 6803 (54%), *Synechococcus* sp. PCC 7942, *Klebsiella pneumoniae* (50%), *Rhizobium leguminosarus* (47%), *Bradyrhizobium japonium* (46%), Azospirillum brasilense (46%), *Rhodobacter capsulatus* (46%) and *Methanococcus* glnB-like protein 1 (35%) (Hsieh *et al.*, 1998).

Between the two plant PII proteins (Caster bean and Arabidopsis), there are 90.3% identity within the region homologous to the microbial PII (Figure 1.5). Another plant PII proteins (Alfalfa) also showed 64% identity to that of *Arabidopsis thaliana.*

Sequence conservation of various PII proteins is most prominent in the signature domains I and II (Hsieh *et al.*, 1998; Jiang *et al.*, 1997). In the signature domain II, the homology among prokaryotes is close to 80% and this region is extremely conserved in *Arabidopsis thaliana* and castor bean PII protein. Such domain may thus play an important role in PII function across all three domain of life. Nevertheless, its exact functions are remained to be explored.

Signature domain I including the uridylylation site Tyr-51 is highly conserved among prokaryotes (Brown *et al.*, 1971; Cheah *et al.*, 1994). However, in

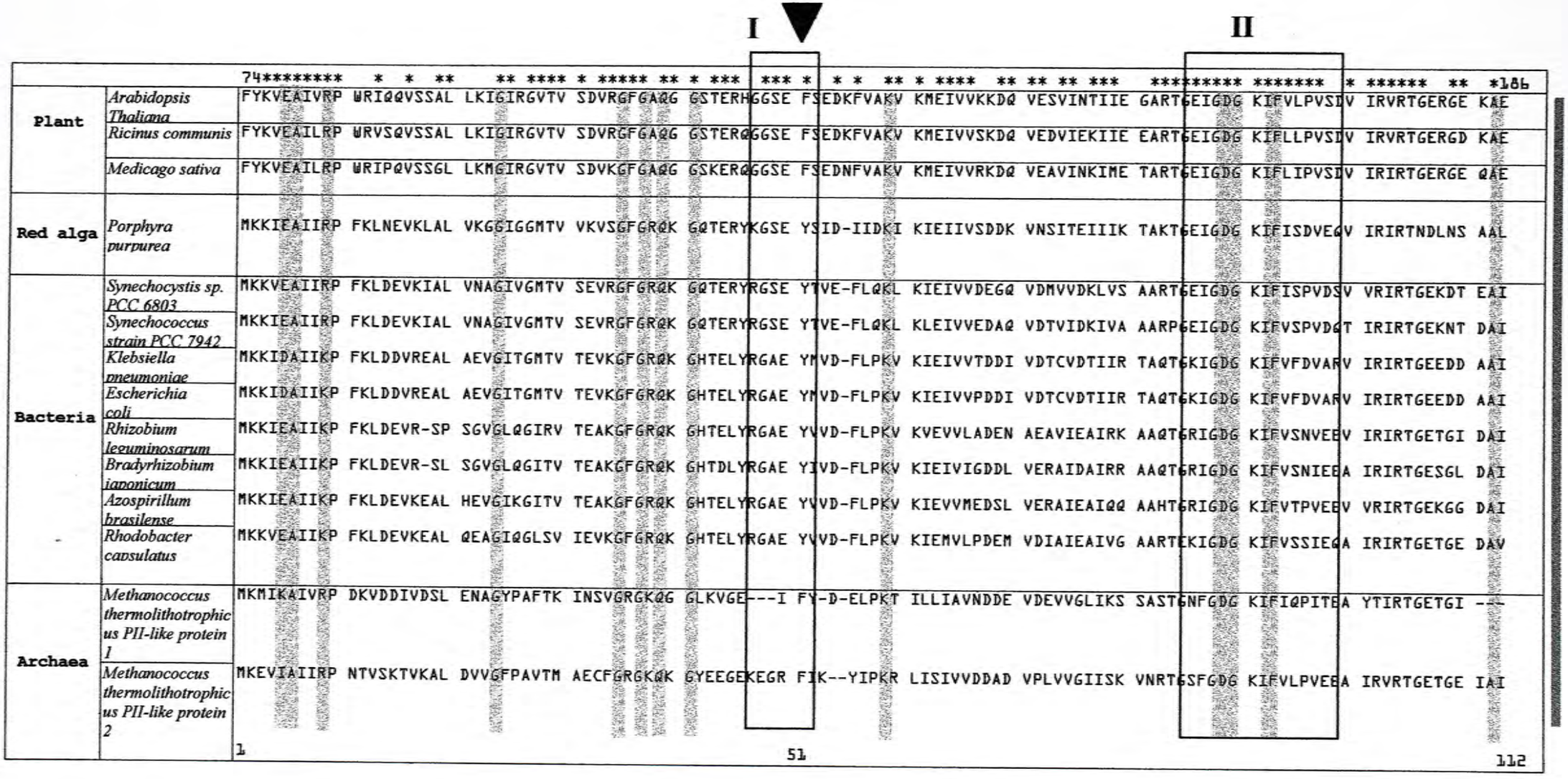
Arabidopsis thaliana, Alfalfa and Castor bean PII protein, the only three plant PII-like proteins identified, such site is replaced by an amino acid phenylalanine (Figure 1.5, Table 1.3). Therefore, plant PII-like proteins may not be uridylylated as that in *E. coli.* Interestingly, both plant PII-like proteins contain a conserved Ser-49 residue. The observation coincides with the study in cyanobacteria PII that modification is via phosphorylation at Ser-49 (Forchhammer & de Marsac, 1994; Hsieh *et al.*, 1998).

1.6 Nitrogen assimilation in higher plants

1.6.1 Nitrogen uptake

Nitrogen assimilation is a vital process controlling the plant growth and development. In plant, all inorganic nitrogen should be first reduced to ammonia before it is incorporated into organic form that will be further metabolized (Crawford & Arst Jr., 1993; Lam *et al.*, 1996). Except in legume plants that can convert atmospheric N_2 into NH_4^+ via symbiotic nitrogen fixation, inorganic nitrogen in form of nitrate in soil is first taken up by plants and subsequently reduced to ammonia via the combined activities of nitrate reductase and nitrite reductase.

Figure 1.5: Comparison of the deduced amino acid sequences of plant and microbial PII-like polypeptides. The asterisks (*) indicate residues that are shared between the plant sequences and any prokaryotic PII-like proteins. Box I and box II refer to the PII signature domains I and II. "▼" indicated the tyrosine residue that is uridylylated in *E. coli* (Hsieh *et al.*, 1998).



Plant	*Arabidopsis Thaliana*	FYKVEAIVRP WRIQQVSSAL LKIGIRGVTV SDVRGFGAQG GSTERHGGSE FSEDKFVAKV KMEIVVKKDQ VESVINTIIE GARTGEIGDG KIFVLPVSDV IRVRTGERGE KAE
	Ricinus communis	FYKVEAILRP WRVSQVSSAL LKIGIRGVTV SDVRGFGAQG GSTERQGGSE FSEDKFVAKV KMEIVVSKDQ VEDVIEKIIE EARTGEIGDG KIFLLPVSDV IRVRTGERGD KAE
	Medicago sativa	FYKVEAILRP WRIPQVSSGL LKMGIRGVTV SDVKGFGAQG GSKERQGGSE FSEDNFVAKV KMEIVVRKDQ VEAVINKIME TARTGEIGDG KIFLIPVSDV IRIRTGERGE QAE
Red alga	*Porphyra purpurea*	MKKIEAIIRP FKLNEVKLAL VKGGIGGMTV VKVSGFGRQK GQTERYKGSE YSID-IIDKI KIEIIVSDDK VNSITEIIIK TAKTGEIGDG KIFISDVEQV IRIRTNDLNS AAL
Bacteria	*Synechocystis sp. PCC 6803*	MKKVEAIIRP FKLDEVKIAL VNAGIVGMTV SEVRGFGRQK GQTERYRGSE YTVE-FLQKL KIEIVVDEGQ VDMVVDKLVS AARTGEIGDG KIFISPVDSV VRIRTGEKDT EAI
	Synechococcus strain PCC 7942	MKKIEAIIRP FKLDEVKIAL VNAGIVGMTV SEVRGFGRQK GQTERYRGSE YTVE-FLQKL KLEIVVEDAQ VDTVIDKIVA AARPGEIGDG KIFVSPVDGT IRIRTGEKNT DAI
	Klebsiella pneumoniae	MKKIDAIIKP FKLDDVREAL AEVGITGMTV TEVKGFGRQK GHTELYRGAE YMVD-FLPKV KIEIVVTDDI VDTCVDTIIR TAQTGKIGDG KIFVFDVARV IRIRTGEEDD AAI
	Escherichia coli	MKKIDAIIKP FKLDDVREAL AEVGITGMTV TEVKGFGRQK GHTELYRGAE YMVD-FLPKV KIEIVVPDDI VDTCVDTIIR TAQTGKIGDG KIFVFDVARV IRIRTGEEDD AAI
	Rhizobium leguminosarum	MKKIEAIIKP FKLDEVR-SP SGVGLQGIRV TEAKGFGRQK GHTELYRGAE YVVD-FLPKV KVEVVLADEN AEAVIEAIRK AAQTGRIGDG KIFVSNVEEV IRIRTGETGI DAI
	Bradyrhizobium japonicum	MKKIEAIIKP FKLDEVR-SL SGVGLQGITV TEAKGFGRQK GHTDLYRGAE YIVD-FLPKV KIEIVIGDDL VERAIDAIRR AAQTGRIGDG KIFVSNIEEA IRIRTGESGL DAI
	Azospirillum brasilense	MKKIEAIIKP FKLDEVKEAL HEVGIKGITV TEAKGFGRQK GHTELYRGAE YVVD-FLPKV KIEVVMEDSL VERAIEAIQQ AAHTGRIGDG KIFVTPVEEV VRIRTGEKGG DAI
	Rhodobacter capsulatus	MKKVEAIIKP FKLDEVKEAL QEAGIQGLSV IEVKGFGRQK GHTELYRGAE YVVD-FLPKV KIEMVLPDEM VDIAIEAIVG AARTEKIGDG KIFVSSIEQA IRIRTGETGE DAV
Archaea	*Methanococcus thermolithotrophicus PII-like protein 1*	MKMIKAIVRP DKVDDIVDSL ENAGYPAFTK INSVGRGKQG GLKVGE---I FY-D-ELPKT ILLIAVNDDE VDEVVGLIKS SASTGNFGDG KIFIQPITEA YTIRTGETGI ---
	Methanococcus thermolithotrophicus PII-like protein 2	MKEVIAIIRP NTVSKTVKAL DVVGFPAVTM AECFGRGKQK GYEEGEKEGR FIK--YIPKR LISIVVDDAD VPLVVGIISK VNRTGSFGDG KIFVLPVEEA IRVRTGETGE IAI

1.6.2 Primary nitrogen assimilation

Ammonia is assimilated into glutamine and glutamate through the GS/GOGAT cycle similar to that in bacteria (see above). In bacteria, GDH is responsible for 85% nitrogen assimilation when ammonia in the medium is in excess. On the other hand, plant GDH only accounts for about 1-5% of the total nitrogen flux (Rhodes *et al.*, 1986, Rhodes *et al.*, 1989). In plants, GDH may play a catabolic role in the metabolism of protein-derived amino acids by deamination of glutamate to yield α-ketoglutarate (re-enter Krebs cycle) which is particularly important during seed germination, senescence and under stress conditions. (Lea *et al.*, 1990, Stewart *et al.*, 1980). Moreover, GDH may also function to provide the initial dose of glutamate to start the GS/GOGAT cycle (Figure 1.1).

1.6.3 Nitrogen transport and interconversions

The nitrate or ammonium ions uptaken by the plants will be assimilated in root or in shoot after transport via xylem (Lea & Ireland, 1999). Through the GS/GOGAT cycle, ammonia is assimilated into glutamine and glutamate. These two amino acids act as important nitrogen donors that can be further convert into another amino acids by the action of aminotransferases (Givan, 1980). They are also nitrogen source for

nitrogen-containing compounds such as purines, purimidines and chlorophylls.

1.6.4 Nitrogen flow

The path of nitrogen flow in plant cells depends on the availability of carbon and nitrogen, tissue type, and plant species concerned, developmental stages and a variety of environmental factors. A stringent control to regulate the distribution of nitrogen and carbon resources within the plant is very important since amino acid synthesis places a heavy demand of organic carbon to the plants. For instance, large amounts of pyruvate, oxaloacetate and α-ketoglutarate are required for amino acid synthesis (Juppe & Turpin, 1994).

Nitrogen flow is crucial for the plant to allocate resources to the appropriate locations. For example, young leaves require incoming nitrogen for growth whereas mature leaves re-export most of their nitrogen to developing fruits or young leaves. To save resources in senescing leaves, they degrade most of their nitrogen-containing molecules and convert them into transporting compounds that are moved out to storage or growth organs (Ireland & Lea, 1999). The amino acid asparagine is thought to be an important compound for transport and storage of nitrogen resources because of its relative stability and high nitrogen to carbon ratio. It is also one of the major transporting amino acid detected in the phloem exudates (Lam *et al.*, 1995).

1.6.5 Molecular regulation of nitrogen assimilation and the possible roles of PII in plants

Important enzymes involved in the nitrogen assimilation, including glutamine synthetase (GS), glutamate synthase (GOGAT), glutamate dehydrogenase (GDH) and aparagine synthetase (AS), generally occur in multiple isoenzymic forms encoded by small gene families. The corresponding genes for those enzymes are subjected to the control by light as well as carbon and nitrogen metabolites.

There are growing evidences suggesting that plant cells can sense carbon and/or nitrogen status as metabolic signals to control gene expressions. For example, hexokinase is proposed to be the switching enzyme that can sense the carbon availability inside the plant cell (Jang *et al.*, 1997). However, the mechanisms controlling intra- and intercellular transport of inorganic and organic nitrogen in plants and the nitrogen sensor are presently unknown. It is not clear whether plant cells possess separate nitrogen sensor or some overlapping components involved in sensing the internal status of both carbon and nitrogen. Based on the bacteria model, PII is a nitrogen signaling molecules that regulate the GS gene expression and activity in response to the nitrogen/carbon ratio. The newly found PII-like protein in *Arabidopsis thaliana* (Hsieh *et al.*, 1998) may be also involved in similar nitrogen sensing mechanism in plants.

The Arabidopsis PII protein is a nuclear-encoded chloroplastic protein (Hsieh *et al.*, 1998). The *GLB1* gene (encoding for Arabidopsis PII protein) appears as a single or low copy number gene located on the chromosome IV of *Arabidopsis thaliana* (Hsieh *et al.*, 1998). Its gene expression is induced by light and such induction occurs within the first hour of light treatment. Moreover, it is also induced by sucrose and repressed by amino acids such as asparagine, glutamine and glutamate (Table 1.4) (Hsieh *et al.*, 1998). Since light, carbon and nitrogen metabolites appear to regulate and coordinate the expression of nitrogen assimilatory genes (including *NR, GLN2, ASN1, ASN2* and *GLU1)*, it supports the notion that plant PII also play a role in the regulatory mechanism.

To examine the exact role of PII in the nitrogen assimilation process and its effects on plant growth and development, homozygous transgenic plants overexpressing PII or truncated PII were successfully constructed and evaluated (Hsieh *et al.*, 1998). *In planta* bioassay showed that the PII overexpressing transgenic lines are much less effective in reversing the sucrose-induced anthocyanin accumulation by the addition of nitrogen (in form of glutamine), when compared to the wild-type plant (Hsieh *et al.*, 1998). This result suggests that deregulating the PII expression may alter carbon/nitrogen sensing ability and PII protein may be involved in sensing the status of carbon and organic nitrogen in higher plants (Hsieh *et al.*, 1998).

	Light	Dark	Organic C-source: sugar		Organic N-source: amino acid		
			Sucrose (metabolic sugar)	Mannitol	Asparagine	Glutamine	Glutamate
GLB1 expression	Induce	Repress	Induced in dark	No effects	Repressed in light with sucrose supplement		

Table 1.4: The regulation of *PII(GLB1)* gene expression. The expression of Arabidopsis *PII(GLB1)* gene is regulated by metabolites and light. Light and sucrose increase while amino acids reduces levels of *PII(GLB1)* mRNA. (Hsieh *et al.*, 1998)

1.7 Hypothesis of this study

My study is to continue to examine the function of PII protein in *Arabidopsis thaliana* by characterizing the homozygous transgenic plants that overexpressing PII or truncated PII. The hypothesis of my study is that *Arabidopsis thaliana* PII protein plays an important role in the nitrogen assimilation process by its effects on numerous important nitrogen-related metabolisms. Through its influences in various nitrogen-related metabolisms it ultimately affects the growth and development of plants.

To test this hypothesis, the growth and development of PII and truncated PII transgenic lines were investigated and examined. My study would mainly focus on several aspects: (i) record the general growth profiles of the transgenic plants; (ii) examine the effects of overexpressing PII or truncated PII on the growth of different plant tissues including young seedlings (fresh weight as the measuring parameter), leaves (chlorophyll as the measuring parameter), roots (root length as the measuring parameter) and seeds (nitrogen and carbon content of seeds as the measuring parameter) under different nitrogen supply; (iii) Study the changes on expression levels of nitrogen assimilatory genes including *ASN1*, *ASN2*, *ASN3*, *GSL1*, *GSR2*,

NIA1 and *NIA2;* and (iv) determine the overall GS enzyme activities (target of PII in *E. coli*) in the transgenic plants.

2 Materials and Methods

2.1 Materials

2.1.1 Plant materials

Arabidopsis thaliana ecotype Col-0 seeds were from the seed stock in Professor H.M. Lam's laboratory in the Department of Biology at The Chinese University of Hong Kong. Homozygous PII overexpressing transgenic lines (PII24-1T4 and PII1-13T3), homozygous truncated PII overexpressing transgenic lines (TPII6-9T2 and TPII3-6T2), and the empty vector transformant control (359.2A10T3), were all from Professor Gloria Coruzzi's laboratory at New York University. PII overexpressing trangenic lines were constructed by inserting the full-length Arabidopsis *PII (GLB1)* mRNA driven by a constitutively expressed promoter. Truncated PII overexpressing transgenic lines were constructed by inserting the Arabidopsis *PII (GLB1)* mRNA that is truncated at 370bp from the 3' end and driven by a constitutively expressed promoter. Western blot analysis indicated that the protein levels of truncated lines were reduced when compared to wild type control, Col-0.

2.1.2 Equipments and facilities used

All equipments and facilities were provided by Department of Biology at the Chinese University of Hong Kong. Major items involved were tabulated below:

Instruments/machines	Company and catalogue number
1. Biological Safety Cabinet	Baker SG600E 59419
2. Refrigerated Centrifuge	Eppendorf 5810R
3. Centrifuge	Eppendorf 5415C
4. Centrifuge	Eppendorf 5415D
5. Gel 1000UV Fluorescent Gel Doc	Bio-Rad 170-3938
6. Environmentally-controlled growth Chamber for soil grown plants	South China House of Technology (Development) Limited
7. Environmentally-controlled growth Chamber for tissue culture plants	Percival AR-32L 3859-05-971
8. Ultrapure water	Millipore PROG00001
9. CHNS/O Analyzer	Perkin Elmer 2400
10. GS Gene Linker UV Chamber	Bio-Rad 0392-92-0336
11. Rotatory Hybridization Incubator	Shel-lab Model 1004
12. Programmable Thermal cycler	MJ research PTC-100 96VHB 200003879
13. Centrivap concentrator	Labconco 79840-01
14. Amino acid analyzer	Beckman System 6300 and Hitachi L8800
15. 96-well quartz microplate	Molecular Devices R8024
16. Microplate spectrophotometer	Molecular Devices s02334

2.1.3 Growth media

2.1.3.1 Soil

The Metro-mix 200 soil (Hummert 10-0325) was used in all experiments.

2.1.3.2 Growth media and solutions required for tissue culture

All media and solutions listed below were prepared in class II safety cabinet after sterilizing with autoclave chambers.

2.1.3.2.1 Preparation of full strength MS medium

One pack of MS salt (Murashige & Skoog Salt mixture, Gibco-BRL cat. no. 11117-017) was dissolved in about 800ml deionized water. Thirty grams sucrose (AJAX 530-500G) and 0.5g MES (4-morpholineethanesulfonic acid, Boehringer Mammhem 223794) were subsequently added. The pH was adjusted to 5.7 with 1M KOH (AJax 405). Nine grams of bactoagar (Difco Bacto™ Agar 214010) were added before the final volume of the solution was adjusted to 1L, followed by autoclaving for 15-20 minutes (Psi=22lb/in^2, 121°C). After autoclaving, the solution was mixed with a magnetic stirrer (including in the solution before autoclaving), cooled down to around 60°C, before pouring into

square petri-plates (Simport D210-16). The plates were ready to use after the agar solidified. Unused plates were stored at 4°C for a maximum period of one month.

2.1.3.2.2 Preparation of ammonium-free MS medium

Ammonium-free MS medium was prepared using the same procedures of full-strength MS medium, except that ammonium-free MS salt (Sigma cat. no. M-8280) was used instead.

2.1.3.2.3 Preparation of nitrogen-free MS medium

Ten mililiter of 100X Liquid MS Micronutrient salts (GIBCOBRL 11155-025) was supplemented with 0.014mg $CoCl_2.6H_2O$ (Peking Chemical Works), 180.7mg $MgSO_4.7H_2O$ (Ajax 302-500g) and 170mg KH_2PO_4 (Ajax 391) so that the components of this medium was exactly the same as the full-strength MS except that it was free of any inorganic nitrogen. The same procedures for making full-strength MS medium were then adopted. MS formula used in all experiments are described in details in Table 2.1.

2.1.4 Buffers and Solutions used in RNA extraction

To avoid the RNase contamination, all aqueous solutions for RNA experiments except RNA extraction buffer and 10X detection buffer were pretreated with DEPC (diethylpyrocarbonate, Sigma D5758 & Amershan US14710). In all DEPC treatments, 0.1% (v/v) of DEPC was added into the solutions before mixing vigorously to disperse all the DEPC droplets. The DEPC-treated solutions were placed in a chemical hood overnight and then autoclaved to remove the remaining DEPC.

2.1.4.1 RNA extraction buffer

One liter of this buffer was made up of 200mM Tris base (Boehringer Mannhem 604205), 400mM KCl (Sigma P9541), 200mM sucrose (AJAX 530-500G), 35mM $MgCl_2$ (RDH 31413) and 25mM EGTA (Ethylene glycol-bis (beta-aminoethyl ether)-N, N, N', N'-tetraacetic acid, Boehringer Mannhem 1093053). The pH of final solution was adjusted to 9.0 and then autoclaved before use.

2.1.4.2 Phenol/Chloroform/Isoamyl alcohol solvent (25:24:1)

Twenty-five parts of buffer-saturated phenol (GiBCO 15509-037), 24 parts of chloroform (Merck 3445) and 1 part of isoamylalcohol (Merch 100979) were mixed. To prevent oxidation of phenol, a layer of 0.2M Tris solution (pH 7.0) containing

Table 2.1: Comparison of the components of the MS salts mixture used in experiments.

	1X MS salt mixture (Gibco-BRL cat. No. 11117-017) /mg	1X Ammonium-free MS salt mixture (Sigma cat. No. M-8280) /mg	100X Nitrogen-free MS salts (liquid) (Gibco-BRL cat. No. 11155-025) /mg
Ammonium nitrate	1,650.0	-	-
Sodium nitrate	-	1751.0	-
Boric acid	6.2	6.2	620.0
Calcium chloride anhydrous	332.2	332.2	-
Calcium chloride.$6H_2O$	-	-	2.5
Cobalt chloride anhydrous	0.014	-	-
Cobalt chloride.$6H_2O$	-	0.025	-
Cupric chloride anhydrous	0.016	-	-
Cupric chloride. $5H_2O$	-	0.025	2.5
EDTA (Disodium salt)	37.25	-	3,730.0
Ferrous sulfate. $7H_2O$	27.8	27.8	2,780.0
Magnesium sulfate	180.7	180.7	-
Manganese sulfate.H_2O	16.9	16.9	1,690.0
Molybdic acid (Sodium salt). $2H_2O$	0.25	0.25	25.0
Potassium iodide	0.83	0.83	83.0
Potassium nitrate	1900.0	1900.0	-
Potassium phosphate monobasic	170.0	170.0	-
Zinc sulfate. $7H_2O$	8.6	8.6	860.0

0.1% (w/v) 8-hydroquinone was added to the top. The mixture was allowed to settle overnight until the aqueous and organic layers separated, before stored at 4°C for future use.

2.1.4.3 3M Sodium acetate, pH 5.6

An amount of 49.22g of sodium acetate (Sigma S-2889) was mixed with 120ml distill water and the pH was adjusted to 5.6 by 16% HCl (Merck 317.2500). The solution was then brought up to a final volume of 200ml using distill water before the DEPC treatment and autoclaving.

2.1.4.4 3M Sodium acetate, pH 5.2

The same procedures as that of 3M sodium acetate, pH5.6 were used except that the pH was adjusted to pH 5.2.

2.1.4.5 Other chemicals

Absolute ethanol for nucleic acid precipitation was from Merck 100986.

2.1.5 Buffers and solutions used in Northern blot analysis

All aqueous solutions were DEPC-treated to remove ribonuclease and autoclaved

before use (see above).

2.1.5.1 Ribonuclease-free water

DEPC-treated Ultrapure water was used for making all aqueous solutions containing RNA samples.

2.1.5.2 10X MOPS

One liter solution containing 200mM MOPS (Boehringer Mannhem 124684), 50mM sodium acetate and 10mM EDTA· Na_2 ((Ethylenedinitrilo)tetraacetic acid, disodium salt, Boehringer Mannhem 808270) was adjusted to pH 7.0 using solid sodium hydroxide (Merck 6498).

2.1.5.3 20X SSC

One liter solution containing 3M sodium chloride (RDH 31434) and 300mM sodium citrate (Sigma S-4641) was adjusted to pH 7.0 using concentrated hydrochloric acid (Ajax 1364).

2.1.5.4. 10% Blocking reagent

Ten grams of blocking reagent (Boehringer Mannhem 1363514) was mixed with

100ml 1X maleic acid buffer and heat to 60°C until completely dissolved. Unused solutions were stored at 4°C for future use.

2.1.5.5 1M Sodium phosphate, pH 7.0

One hundred milliliter each of 1M $NaH_2PO_4.2H_2O$ (Sodium dihydrogen orthophosphate, Ajax 478) and 1M $Na_2HPO_4.2H_2O$ (Di-sodium hydrogen orthophosphate, Ajax 621) were prepared as stocks. One molar Na_2HPO_4 was titrated with 1M $NaH_2PO_4.2H_2O$ until the pH reached 7.0.

2.1.5.6 10% N-lauroylsarcosine

Ten grams of N-lauroylsacrosine, sodium salt (Sigma L9150) was dissolved in 100 ml of distill water before filtering through a 0.2 μm filter (Schleicher & Schuell FP03013)

2.1.5.7 10% SDS

Ten grams of SDS (Sodium Dodecyl Sulfate, Boehringer Mannhem 1028685) was dissolved in 100ml of distill water.

2.1.5.8 10X maleic acid buffer, pH 7.5

One liter solution containing 1M Maleic acid (Sigma M-0375) and 1.5M sodium chloride was adjusted to pH 7.5 using solid sodium hydroxide (Ajax 482-500G).

2.1.5.9 10X detection buffer

One liter solution containing 1M Tris and 1M sodium chloride was adjusted to pH 9.5 using concentrated hydrochloric acid (Ajax 1364).

2.1.5.10 Other chemicals and reagents used in Northern blot analysis

Materials/ chemicals	Company and catalogue number
Nylon membrane	Boehringer 1417240
3MM Filter paper	Whatman 3030931
Formaldehyde	Sigma F8775
Agarose	GibcoBRL 15510-027
Formamide	Boehringer Mannhem 1814320
Ethidium bromide	Sigma E7637
Loading dye (Bromophenol blue)	Merck 8122
X-ray film	Biomax 870 1302
CSPD (Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyco[3.3.1.1^{3,7}]decan}-4-	Boehringer 1755-633

yl)phenyl phosphate)	
RNaseZap	Sigma R-2020
Anti-digoxigenin-AP (Anti-DIG)	Boehringer 1093274

2.1.6 Molecular reagents and synthetic oligonucleotides used in the preparation of DIG-labeled probes

2.1.6.1 Reagents

Reagents	Company and catalogue number
10X PCR buffer with 15mM $MgCl_2$	Roche 164679
25mM $MgCl_2$	Roche 164679
1mM dNTPs	Roche 196064
Taq DNA polymerase	Roche 1647679
1mM DIG dNTPs	Boehringer Mannhem DIG DNA Labeling Kit 1175033
Forward primers (20-mers)	Life Technologies
Reverse primers (20-mers)	Life Technologies

2.1.6.2 Primer sequences

In some cases, more than one pair of primers were designed and used for amplification. In general, the longest probe for each gene was used in the hybridization experiments.

2.1.6.2.1 Primers for PII (GLB1) gene:

No.	Type of primer	sequences
HMOL 559	Forward primer	5'AACTAGAATCATGGCGG3'
HMOL 560	Forward primer	5'CCGAGTAATAACAGTCGT3'
HMOL 561	Reverse primer	5'GAAACCAAACACAGACTCC3'

PCR products resulting from HMOL 559 & HMOL 561 spans a region of 692bp close to the 5'-end of the *PII (GLB1)* gene.

PCR products resulting from HMOL 560 & HMOL 561 spans a region of 535bp close to the 5'-end of the *PII(GLB1)* gene.

2.1.6.2.2 Primers for GSL1 gene:

No.	Type of primer	Sequences
HMOL 675	Forward primer	5'TTGAACCTCTCGCTTCGCCA3'
HMOL 676	Reverse primer	5'ACATTGTCCATACAATTCTG3'
HMOL 677	Forward primer	5' GAGCCAAACTCTTGAGGCTGA3'
HMOL 678	Reverse primer	5' GGACATGCTCTAACAGTCAA3'

PCR products resulting from HMOL 675 & HMOL 676 spans a region of 446bp close to the 3'-end of the *GSL1* gene.

PCR products resulting from HMOL 675 & HMOL 678 spans a region of

488bp close to the 3'-end of the *GSL1* gene.

PCR products resulting from HMOL 677 & HMOL 678 spans a region of 236bp close to the 3'-end of the *GSL1* gene.

2.1.6.2.3 Primers for GSR2 gene:

No.	Type of primer	sequences
HMOL 690	Forward primer	5'CCATCGGTCGGTATCTCAGC3'
HMOL 691	Reverse primer	5'AACTCCGGCAGTGTCAACCG3'
HMOL 692	Reverse primer	5'GGCCTTTTCAGATTGTATTA3'

PCR products resulting from HMOL 690 & HMOL 691 spans a region of 579bp close to the 3'-end of the *GSR2* gene.

PCR products resulting from HMOL 690 & HMOL 692 spans a region of 719bp close to the 3'-end of the *GSR2* gene.

2.1.6.2.4 Primers for NIA1 gene:

No.	Type of primer	sequences
HMOL 651	Forward primer	5'GTTGGTCATATCGACCTCG3'
HMOL 652	Reverse primer	5'TTATGCTTACTAGCCCATCC3'

PCR products resulting from HMOL 595 & HMOL 959 spans a region of 365bp close to the 3'-end of the *NIA1* gene.

2.1.6.2.5 Primers for NIA2 gene:

No.	Type of primer	sequences
HMOL 649	Forward primer	5'ACACCAAGCAGCACCGTTGA3'
HMOL 650	Reverse primer	5'CCATCCAGTTCCTCCCTTAG3'

PCR products resulting from HMOL 649 & HMOL 650 spans a region of 370bp close to the 3'-end of the *NIA2* gene.

2.1.7 Chemicals used in BioRad Protein Assay

2.1.7.1 Preparation of the BioRad protein assay standard

A stock (1.45mg/ml) of protein assay standard was prepared by dissolving the lyophilized bovine gamma standard (Bio-Rad 500-0007) in 20ml of deionized water, before aliquoted and stored at -20°C. A 1mg/ml working solution was prepared by mixing 10ml of the stored stock with 4.5ml distill water.

2.1.7.2 Dye

One part of dye concentrate (Bio-Rad 500-0002) was mixed with four parts of distill water, before filtered through Whatman No.1 filter paper and stored at room temperature before use.

2.1.8 Chemicals and apparatus used in chorophylls extraction & quantitation

Reagents / apparatus	Company and catalogue number
N, N-dimethylformamide (DMF)	Sigma D-4551
96-well quartz curette	Molecular Devices R8024

2.1.9 Buffers and solutions used in the glutamine synthetase enzyme extraction and assay

2.1.9.1 GS extraction buffer

The final concentration of chemical components included 50mM Imidazole (Sigma I2399), 10mM $MgCl_2$ (RDH 31413) and 14.3M β-mercaptoethanol (Sigma M-6250). The pH was adjusted to 7.5 using concentrated HCl (Ajax 1364). β-mercaptoethanol was added to the solution right before use.

2.1.9.2 GS assay buffer

The final concentration of chemical components included 30mM L-glutamine (Sigma G8783), 40mM imidazole (Sigma I2399), 3mM $MnCl_2$ (Ajax 307), 0.4mM NaADP (Sigma A2754), 10mM Na_2HAsO_4 (Sigma A-6631) and 80mM hydroxylamine (Sigma H2391). The solution was stored at -20°C before use.

2.1.9.3 Stop solution

Two and a half milliliter of concentrated HCl was mixed to 117.5ml distilled water, before further addition of 4g $FeCl_3$ (RDH 12321) and 2.4g trichloroacetic acid (Sigma T-4885). The resulting solution was mixed until dissolved and filtered to remove undissolved residue. The stop solution was stored, if necessary, at room temperature.

2.2. Methods

2.2.1 Plant growth

2.2.1.1 Surface sterilization of seeds for growth on agar plates

Arabidopsis thaliana seeds were put into microfuge tubes (1.5-2.0ml) and surface sterilized with 1ml chlorox (5.25% hypochlorite) for 3 minutes with shaking and vortexing. Special care was needed to ensure well separation of the seeds. Chlorox solution was removed by aspiration. After centrifuged briefly, the seeds were then rinsed in 1ml sterilized water for 3 times, by mixing and centrifugation. Finally, a few drops of sterilized water were added to the washed seeds before sown on agar plates.

2.2.1.2 Plants grown on MS plates with different supplements

In a class II safety cabinet, surface sterilized seeds were sown on nylon meshes (Tikko) placed on full strength MS (Murashige & Skoog Salt mixture, Gibco-BRL) agar square plates. About 30 seeds were placed separately in a horizontal line per square plate. The plates were then sealed with parafilm and the seeds were imbibed at 4°C in dark for 1 to 2 days. After imbibition, the plates were placed vertically in growth chambers with regular light/dark cycle (16 hours light and 8 hours dark) at 22°C for 14 days. The light intensity was around 80-100μE. Subsequently, sterilized forceps were used to transfer the nets, in which the seedlings were transferred to new plates with designed formula. For continuous darkness, the plates were covered with two layers of foil and placed inside the dark compartment of growth chambers. For continuous light, the dark period settings of the growth chambers were deleted.

2.2.1.3 Preparation for soil growth

Surface sterilized seeds were sown and grown on the full strength MS (Murashige & Skoog Salt mixture, Gibco-BRL) square plates as described above.

Dry Metro-mix 200 soil was first mixed thoroughly with water. Moist soil was then put into plastic pots placed on a large plastic tray. Eleven to 14-day-old seedlings

with similar root lengths were chosen and transferred to the pre-wet soil. For each experiment, at least 12 seedlings were grown for each line. The seedlings were allowed to grow for another 15 days, followed by appropriate light-dark treatments, before harvesting for RNA or protein.

2.2.2 RNA Extraction

2.2.2.1 Phenol: Chloroform: Isoamylalcohol solvent extraction

Plant tissues were quickly harvested, frozen in liquid nitrogen and ground into fine powder in pre-chilled mortars and pestles. Equal volume of extraction buffer (about 5ml per gram fresh tissues) and phenol: chloroform: isoamyl alcohol (P:C:I) (25:24:1) solution were added. The thawed samples were transferred to 50ml falcon tubes and mixed thoroughly before centrifugation at 8,000g for 5 minutes. The upper aqueous layer was aspirated into a new falcon tube and extracted again with equal volume of P:C:I (25:24:1) solution. After centrifuging at 8,000g for 5 minutes, the upper aqueous layer was transferred to a new falcon tube. One portion of chloroform: isoamyl alcohol (C:I) (24:1) solution was added to remove the residual phenol. After mixing thoroughly, the sample was again aspirated into a new falcon tube. One-tenth volume of 3M sodium acetate (pH 5.2) and 2.5 volume of absolute ethanol were added to the aqueous solution to precipitate all nucleic acids. The sample was then

stored at -20°C overnight before centrifuged at 13,000g for 20 minutes. After removal of the supernatant, the precipitate was resuspended in 1ml 3M sodium acetate (pH5.6) and the suspension was transferred to microcentrifuge tubes (1.5-2.0ml) and centrifuged at 14,000g for 10 minutes. The supernatant was removed and the pellet was resuspended again in 1ml 3M Sodium acetate (pH5.6) and centrifuged. The resulting pellet, after removal of supernatant, was resuspended in 0.4ml 0.3M Sodium acetate (pH5.6). One milliliter of absolute ethanol was then added and the sample was mixed by inverting. The solution was stored at -70°C for at least 20 minutes to allow RNA precipitation. Ethanol was removed by centrifugation at 14,000g for 20 minutes. The pellet, now contained mainly mRNA and rRNA, was dried in the CentriVap concentrator for about 5-10 minutes. The final RNA pellet was resuspended in DEPC-treated water (the final concentration of total RNA was 0.5-10μg/μl) and stored at -70°C.

2.2.2.2 Quantity and quality of RNA samples

One microliter of resuspended RNA was mixed into 400μl DEPC-treated water. Optical densities at 260nm and 280nm were measured. The concentration of RNA was calculated based on the reading of OD_{260nm} (i.e. 1 O.D.=37μg/ml). The samples were considered as in good quality when their 260nm/280nm ratio were close to 2.

Concentration and quality of RNA samples were further evaluated by running 2-5μg aliquots in a denaturing gel.

2.2.3 Northern blot analysis

Northern blot analysis (Sambrook *et al.*, 1989) was performed to investigate the steady-state mRNA level in the transgenic plants.

2.2.3.1 Preparation of DIG-labeled probes

DIG-labeled RNA probes and DIG-labeled single-stranded PCR probes were used in various experiments. Expressions of seven genes including GLB1, *ASN1*, *ASN2*, *GSL1*, *GSR2*, *NIA1* and *NIA2* were examined. RNA probes of *ASN2* and single-stranded DNA probes of *ASN1* were obtained from colleagues in Prof. H. M. Lam's lab.

To make DIG-labeled single-stranded PCR probes (Finckh *et al.*, 1991), two rounds of PCR were performed. In the first round, the reaction mixture contained the following components: 5μl of 10X PCR buffer, 1μl of 25mM $MgCl_2$, 2μl of 1mM dNTPs, 1.7μl of 1.5μM forward primer, 1.7μl of 1.5μM reverse primer, 1μl of DNA templates (1pg/μl), and 36.6μl of distilled water. One microliter of *Taq* DNA polymerase was finally added to make up a final volume of 50μl.

Five microliter of first round PCR product was quantified by gel electrophoresis. Reaction mixture of round two PCR reaction contained the following components: 10 microliter of 10X buffer, 2μl of 25mM $MgCl_2$, 2μl of 1mM DIG dNTPs, 6.6μl of 1.5μM of reverse primer, 5μl of round one DNA (approximately 50ng), 72.4μl of distilled water. Two microliter of Taq DNA polymerase was finally added to make up a final volume of 100μl. After two rounds of PCR, DIG-labelled single-stranded anti-sense DNA probes were generated.

The following PCR program was used for both round 1 and round 2 PCR reactions:

Step 1: 94°C, 2mins

Step 2: 94°C, 20s

Step 3: 53°C, 30s

Step 4: 72°C, 2mins

Step 5: Repeat Step 2 to Step 4 for 54 more cycles

Step 6: 72°C, 10mins

Step 7: 4°C, hold

2.2.3.2 Estimation of probe concentrations

Prior to use, quantity of each DIG-labeled probe was estimated. Serial dilution was performed to obtain 1:5, 1:50, and 1:500 diluted samples. One microliter of each

dilution solution was dotted onto a positively charged nylon membrane. In order to estimate the probe quantities, the DIG-labeled control DNA (5ng/μl) was similarly diluted and dotted onto the same membrane. The membrane was UV crosslinked (total=250mJ) and then washed with 1X maleic acid buffer (pH 7.5) for 2 minutes. The washing buffer was discarded and 2% blocking solution was added to the membrane and incubated for 5 minutes. Subsequently, 1% blocking solution containing 1:10,000 anti-DIG antibody was added and incubated for 10 minutes. The membrane was then washed with 1X maleic acid, pH7.5 for 5 minutes for 2 times. Detection buffer was added after removal of the wash buffer and incubated for 1 minute. After all washings, the membrane was put onto a polyethylene sheet and about 10μl of substrate (CSPD) was added to cover the whole membrane. The membrane was wrapped with a polyethylene sheet and put into X-ray film cassette. Autography was performed at 37°C for 15 minutes using Biomax X-ray film. By comparing the intensity of the dots to the controls, the probe concentration was estimated.

2.2.3.3 Preparation of RNA denaturing gel

For making 100ml RNA denaturing gel, 1g of agarose was added into 87ml DEPC-treated water and the mixture was heated to dissolve in a microwave oven for about

1-2 minutes. The solution was allowed to cool down to about 70°C. Three ml 37% formaldehyde and 10ml 10X MOPS (pH 7.0) were then added and the solution was mixed well. The gel was set and allowed to solidify. One liter running buffer was prepared by mixing 100ml 10X MOPS with 900ml DEPC-treated water.

2.2.3.4 RNA sample denaturation

Same amounts of RNA samples (10-20μg) were added to 3.5μl 10X MOPS, 17.5μl formamide and 6.13μl 37% (w/v) formaldehyde. DEPC-treated water was added to make up the volume to 35μl. 1μl ethidium bromide and 1μl 6X loading buffer (with bromophenol blue as dye) were then added to the mixture. The sample was denatured at 55°C for 20 minutes and then placed on ice for about 2 minutes. Condensation was spun down by a brief centrifugation. The samples were then loaded onto the gel prepared as described above and electrophoresed at 100V for about 1 hour.

2.2.3.5 Capillary Blotting

After electrophoresis, the gel was washed with DEPC-treated water. The area of gel containing samples (with 1cm boundary) was carefully cut out using a razor. An RNase-free glass tank was filled with about 400ml 10X SSC. Two pieces of 3MM paper was cut in appropriate size, soaked in 10X SSC in the glass tank and acted as a

buffer bridge. The gel was put onto the buffer bridge with the bottom side face upward. A polyethylene sheet was placed around the gel to ensure that the buffer from the glass tank would pass through the gel. A nylon membrane, with the same size as the gel, was laid onto the gel after rinsed in 10X SSC. Gas bubbles trapped between the gel and the membrane were removed by rolling a plastic pipette across the membrane. Four pieces of 3MM filter paper, with the same size as the gel, were laid onto the nylon membrane. Gas bubbles were again removed. About five inches of blotting tissue paper was added onto the top and a square glass plate was used to apply weight to ensure good contact between the nylon membrane and the gel. A bubble leveler was used to adjust the level. This blotting set up was left at room temperature for at least 16 hours.

2.2.3.6 Prehybridization and hybridization

After capillary blotting, the corresponding position of wells on the membrane was marked to locate the orientation of samples. The membrane was UV crosslinked (total 250mJ) before washed with DEPC-treated water to remove excessive 10X SSC buffer. The membrane was then transferred to a hybridization tube containing at least 5ml prehybridization solution (2.5ml 100% formamide, 1.75ml 20X SSC, 2ml 10% (w/v) blocking solution, 0.25ml 1M sodium phosphate, pH 7.0 and 37.5μl 10% N-

lauroylsarcosine and 0.35g SDS) and the hybridization tube was put into a rotatory hybridization oven at 42°C for two to four hours.

Hybridization solution was prepared by adding 25μl/ml probes to fresh prehybridization solution. After removal of prehybridization solution, the hybridization tube was added to the hybridization tube containing the nylon membrane. Hybridzation was carried out in a hybridization oven at 42°C for at least 16 hours.

2.2.3.7 Post-hybridization washes

After hybridization, the membrane was first washed in cold wash solution (5ml 20X SSC, 0.5ml 10% SDS and 44.5ml DEPC-treated water) twice at room temperature for 15 minutes each.

The membrane was then transferred to hot wash solution (0.25ml 20X SSC, 75μl 10% SDS and 9.6ml DEPC-treated water) placed in a hybridization tube. The hot wash was performed in a rotatory hybridization oven at 68°C twice for 15 minutes each.

2.2.3.8 Detection of DIG

The washed membrane was transferred to a falcon tube and rinsed with 7.5ml 1X100mM maleic acid buffer. Subsequently, the membrane was blocked in 10ml 2% blocking solution. Eight millilter of 1X maleic acid buffer and 2ml 10% blocking solution stock for 4 hours at room temperature. Ten milliliter of blocking solution containing 1:10000 anti-DIG antibody (8ml 1X maleic acid buffer, 2ml 10% blocking reagent and 1μl anti-DIG stock) was added to the membrane and incubated for 30 minutes at room temperature. The membrane was then washed with 1X maleic acid buffer at room temperature for 15 minutes for two times before soaked in 1X detection buffer at room temperature for 2 minutes. The membrane was put onto a polyethylene sheet. CSPD was added to the nylon membrane and autography was performed using Biomax X-ray film. The film was developed in developer for 2 minutes followed by a rinsing in water. It was then treated with fixer for 3 minutes.

2.2.3.9 The signal-enhanced procedure

.In order to enhance the northern blot signal, a signal-enhanced procedure was performed in which the film developing time was prolonged to 5 minutes.

2.2.4 Chlorophyll extraction and quantitation

Chlorophyll content was determined by spectrophotometric analysis using N,N-dimethylformamide as the solvent (Inskeep & Bloom, 1985; Moran, 1982; Moran & Porath, 1980). For samples grown in soil, two or three rosette leaves were cut and soaked in 800μl DMF in dark at 4°C overnight. For seedlings grown on agar plates, 4 seedlings each were soaked in 500μl DMF in dark at 4°C overnight. Optical density at 603, 625, 647 and 664nm were then measured.

Chlorophyll content was calculated according to the following equations (Moran, 1982):

$$Ca=12.81A_{664}-2.16A_{647}+1.44A_{625}-4.91A_{603}$$

$$Cb=-4.93A_{664}+26.01A_{647}+3.74A_{625}-15.55A_{603}$$

$$Cp=-2.52A_{664}-0.79A_{647}+36.55A_{625}-27.08A_{603}$$

Ca, Cb and Cp represent chlorophyll a, Chlorophyll b and prochlorophyll, respectively. Total chlorophyll content was calculated as the sum of chlorophyll a, chlorophyll b and prochlorophyll and was expressed in μg per seedling. At least duplicated samples were used in one experiment and all experiments were repeated at least twice.

2.2.5 Root length measurement

The seeds were surface sterilized and sown on MS plates with different supplements as described in the Materials and Methods section. The root lengths of 10-day-old seedlings were measured by a ruler. There were at least 30 seedlings for each sample point and the data were analyzed by ANOVA.

2.2.6 Total glutamine synthetase enzyme assay

Total glutamine synthetase enzyme activities were estimated by the transferase assay method (Rhee *et al.*, 1985).

2.2.6.1 glutamine synthetase enzyme assay

Total glutamine synthetase enzyme activity was measured with the transferase method (O'Neal & Joy, 1973; Rhee *et al.*, 1985; Stadtman *et al.*, 1979). About 1 g of sample was ground in a mortar containing 5ml extraction buffer. After the tissue was thoroughly ground, crude enzyme extract was obtained after removal of undissolved residues by centrifugation at 8,000g for 10 min at 4°C. At all time during the extraction, the samples were kept on ice. Fifty microliter of the crude extract was saved for Bio-Rad protein assay.

In transferase assay, 100μl of crude extract was mixed with 200μl of GS assay buffer and incubated for 30 minutes at 37°C. At least two replicates of each sample were assayed. Fifty μl distill water was used as the blank. After the 30-minute incubation, 1ml of stop solution was added. Optical density at 540nm was measured for all samples. Specific enzyme activities were obtained by normalization of total protein contents estimated by Bio-Rad protein assay.

2.2.6.2 Protein quantitation by BioRad protein assay

The protein concentration was estimated by the BioRad protein assay method (Bradford, 1976).

2.2.6.2.1 Standards

A series of standards with different concentrations as 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0mg/ml were prepared and diluted from the 1mg/ml BSA solution. Ten microliter of each concentration was added with 500μl of diluted dye and stand for 15 minutes at room temperature before OD_{595} measurement. A standard curve was drawn from the data.

2.2.6.2.2 Samples

Four to ten microliter of samples was added with 500μl of diluted dye and stand

for 15 minutes at room temperature before OD_{595} measurement. Protein concentration of each sample can be estimated based on the standard curve.

2.2.7 Measurement of total nitrogen in seeds

The percentage of nitrogen and carbon was measured using a CHN/S analyser. For each sample, 100 seeds were used. Two duplicates were done for every sample in each experiment and each experiment was repeated once.

2.2.8 Recording growth and development

The seedlings were first grown on full strength MS agar plates as described in Materials and Methods section before transferred to soil. No additional fertilizers were added to the soil. Photos were then taken for every 2-4 days to record the progress of growth and development of each line.

3. Results

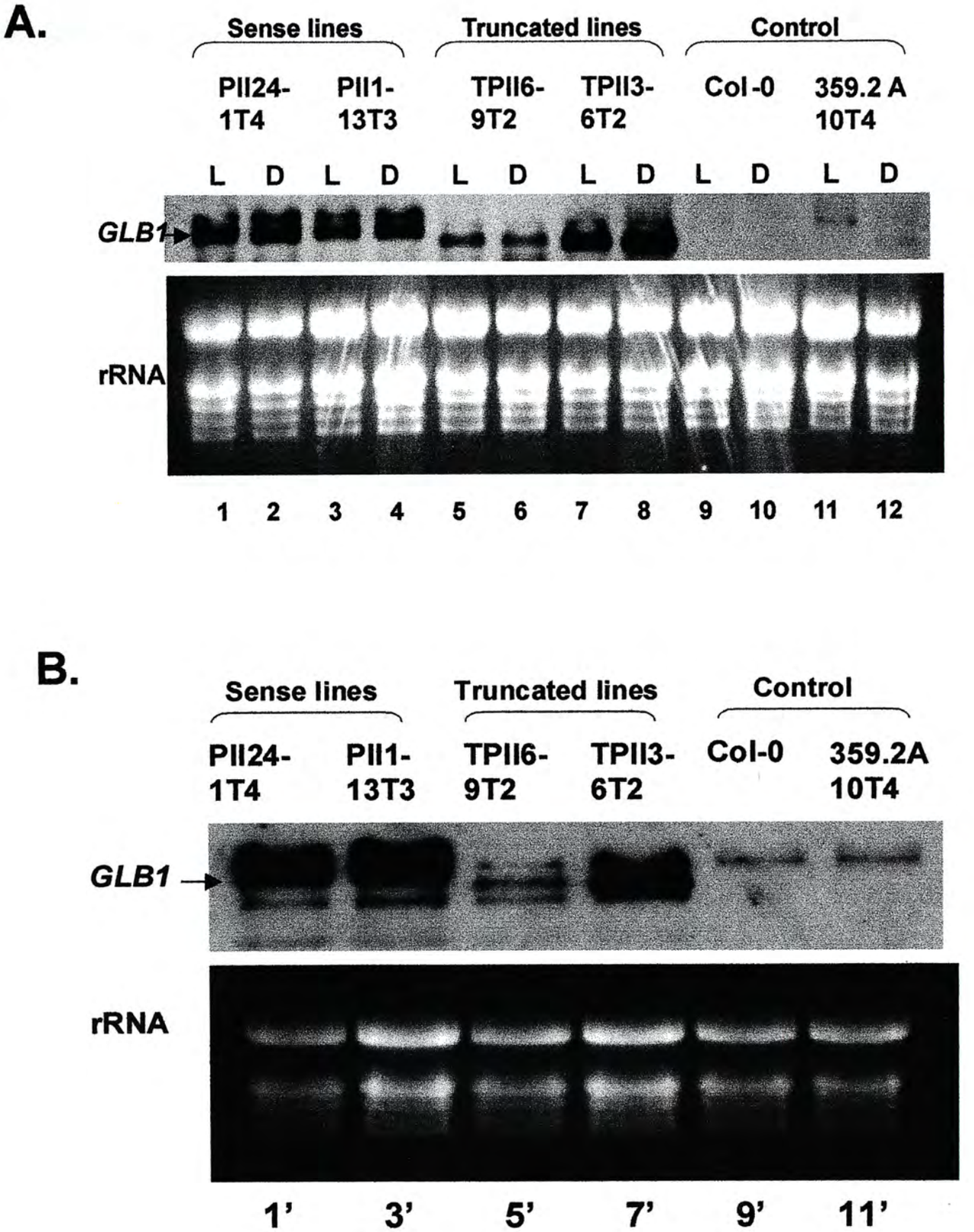
3.1 Overexpression of PII and truncated PII mRNA in transgenic plants

The relative levels of *PII* (*GLB1*) mRNA and truncated *PII (GLB1)* mRNA levels in the transgenic lines were compared to the wild type parent Col-0 and the empty-vector transformant control 359.2A10T3 (See Materials and Methods). Total RNA from rosette leaves were extracted from 26-day-old plants grown on soil. Northern blot analyses showed that the levels of *PII (GLB1)* mRNA in the wild type Col-0 and the control 359.2A10T3 were maintained at low levels and were slightly induced by the light treatment (Figure 3.1A, lanes 9 & 11). Since the signal of lanes 9 and 11 in Figure 3.1A were weak, a separate Northern blot experiment was done using larger combs and longer film exposure time to enhance the signals. A sharp band representing the native *PII (GLB1)* mRNA was observed in both Col-0 and 359.2A10T3 (Figure 3.1B, lanes 9' and 11').

PII (GLB1) mRNA was barely detectable in both controls grown under continuous dark treatment (Figure 3.1, lanes 10 & 12). These results were consistent with the previous findings on the native *PII (GLB1)* gene regulation (Hsieh *et al.*, 1998).

By contrast, *PII (GLB1)* mRNA was constitutively expressed in high levels in the PII

Figure 3.1: *PII (GLB1)* mRNA levels in soil-grown PII and truncated PII transgenic lines. Northern blot analyses were performed using regular (A) or signal enhanced (B) procedures (See Materials and Methods). Eleven-day-old seedlings of the PII transgenic lines PII24-1T4 (lanes 1, 1' and 2) and PII1-13T3 (lanes 3, 3' and 4), the truncated PII transgenic lines TPII6-9T2 (lanes 5, 5' and 6) and TPII3-6T2 (lanes 7, 7' and 8), the wild type Col-0 parent Col-0 (lane 9, 9' and 10) and the empty vector transformant control 359.2A10T3 (lanes 11, 11' and 12) grown on MS agar plates under a regular day-night cycle (16 hours light, 8 hours dark) were transferred to soil for further growth for 14 days. Plants were subsequently treated under continuous light (lanes 1, 1', 3, 3', 5, 5', 7, 7', 9, 9', 11, 11') or continuous dark (lanes, 2, 4, 6, 8, 10, 12) for 48 hours before harvesting. Total leaf RNA was extracted as described in Materials and Methods. An aliquot of 15μg total RNA from each line was applied to the gel. DIG-labeled probes were used to detect the levels of *PII (GLB1)* mRNA (see Materials and Methods). L: light-grown plants; D: dark-adapted plants.


A.
Sense lines
Truncated lines
Control
PII24-1T4
PII1-13T3
TPII6-9T2
TPII3-6T2
Col-0
359.2 A 10T4
L D L D L D L D L D L D
GLB1
rRNA
1 2 3 4 5 6 7 8 9 10 11 12
B.
Sense lines
Truncated lines
Control
PII24-1T4
PII1-13T3
TPII6-9T2
TPII3-6T2
Col-0
359.2A 10T4
GLB1
rRNA
1' 3' 5' 7' 9' 11'

transgenic lines, PII24-1T4 and PII1-13T3 (Figure 3.1A, lanes 1, 2, 3 and 4, Figure 3.1B, lanes 1' and 3') regardless of light or dark treatment. The *PII (GLB1)* mRNA in the dark-adapted PII transgenic lines was as abundant as that detected in the light-grown PII transgenic lines. Interestingly, three discrete bands were found in all PII and truncated PII transgenic lines instead of one sharp band detected in Col-0 and 359.2A10T3 (Figure 3.1).

Based on the results of the control lines, the middle band of PII24-1T4 and PII1-13T3 (PII transgenic lines) and the upper band of TPII6-9T2 and TPII3-6T2 (truncated PII transgenic lines) were presumably corresponding to the intact *PII (GLB1)* mRNA. The sense lines were clearly overexpressing the *PII (GLB1)* mRNA. In the two truncated PII transgenic lines (TPII6-9T2 and TPII3-6T2), the second band of size smaller than intact *PII (GLB1)* was clearly overproduced. Additional bands in the transgenic lines might be due to aberrant mRNA species accumulated in the overexpressing lines. Surprisingly, it was found that one truncated line (TPII6-9T2) showed different levels of truncated *PII (GLB1)* mRNA when grown in MS agar plates, compared to soil. While truncated *PII (GLB1)* mRNA was overproduced in TPII6-9T2 under both light and dark treatment (Figure 3.1A lanes 5 and 6, Figure 3.2 lanes 5 and 6), expression of truncated PII mRNA in TPII6-9T2 was significantly lower than that of TPII3-6T2 in soil-grown plants (Figure 3.1A lanes 5-8). However,

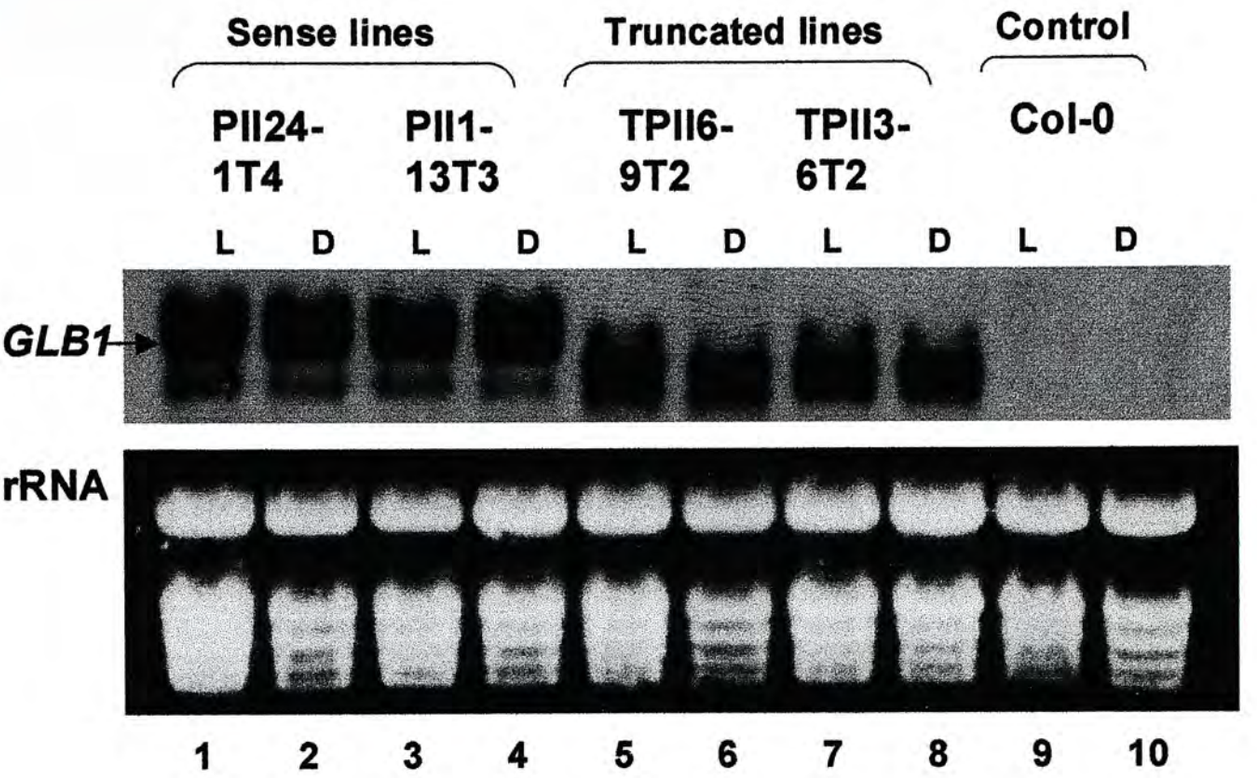


Figure 3.2: *PII (GLB1)* mRNA levels in PII and truncated PII transgenic lines grown on MS agar medium. Northern blot analyses were performed as described in Figure 3.1 except that after the plants were grown on MS agar plates under a regular day-night cycle (16 hours light; 8 hours dark) for 14 days, they were immediately treated under continuous light (lanes 1, 3, 5, 7, 9 and 11) or continuous dark (lanes 2, 4, 6, 8 and 10) for 48 hours before harvesting.

when the plants were grown in MS agar plates, such difference was no longer observed (Figure 3.2, lanes 5, 6, 7 and 8).

3.2 General growth characteristics of PII transgenic plants when grown on soil

General growth characteristics over an extend time span (Figure 3.3 A-D) of PII and truncated PII transgenic lines were observed and recorded. Ten-day-old seedlings from the MS agar plates were transferred to soil for growth. The growth of at least three individuals for each line were observed and recorded. Consistent results were obtained for 3 generations. Initially, the growth rate of PII transgenic lines did not shown obvious differences compared to the wild type Col-0 and empty vector transformant control 359.2A10T3 (Figure 3.3 A-C). However, the mature plants of the PII transgenic lines seemed to be of slightly larger size and thicker stems (Figure 3.3D).

By contrast, both truncated lines seemed to show impaired growth in different forms. TPII6-9T2 bolted much early than controls (Figure 3.3A) and gave thinner stems and small-size leaves when grown in soil (Figure 3.3A-D). The phenotype strongly suggested that TPII6-9T2 was under physiological stress under such growth conditions.

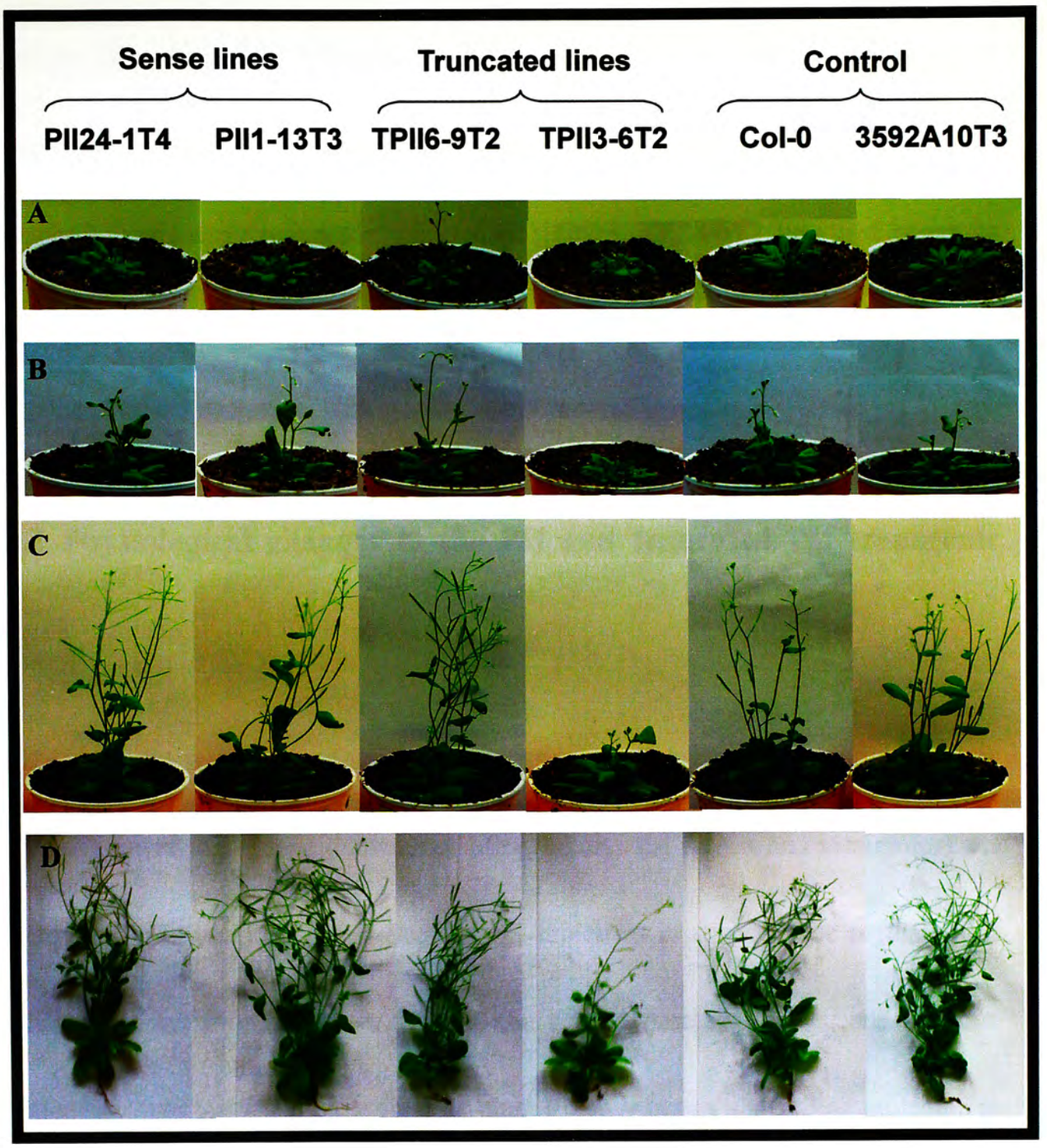


Figure 3.3: General growth characteristics. Ten-day-old seedlings of each line of homogenous sizes were transferred from MS agar plates to soil for 10 (A), 20 (B), 35 (C) and 60 (D) days. The experiment was repeated 3 times with at least 3 individuals for each line and consistent results were obtained.

On the other hand, the other truncated PII transgenic line TPII3-6T2 grew much slower than control (Figure 3.3) with delayed flowering time. When other plants had reached their maturity and formed mature siliques, TPII3-6T2 was just begun to produce its first batch of siliques. Different from TPII6-9T2, however, TPII3-6T2 produced leaves and stems with similar size compared to controls.

3.3 Physiological changes in the PII and truncated PII transgenic lines

PII is thought to be one of the most widespread and highly conserved regulatory proteins found in all three domains of life (prokaryotes, archea and eukaryotes). As described in Literature Review Section, PII protein is involved in the regulation of nitrogen assimilation, nitrogen fixation and related processes in various bacteria. Therefore, it is highly possible that the plant PII may act as a signaling molecule in the nitrogen assimilation. To examine the effect of PII in the nitrogen assimilation, different plant tissues of the PII and truncated PII transgenic plants were analyzed. Furthermore, different nitrogen supplements may result in different effects on the functioning of the PII protein (observed in most bacteria). Plants harvested under different exogenous nitrogen supply were examined and compared to observe if any imbalance of the nitrogen assimilation resulted from overexpressing PII or truncated

PII would lead to a defected or abnormal growth. Since there is not much information on the functions and actions of PII in plants, growth of different plant tissues including leaves, root and seeds of the transgenic lines under different exogenous nitrogen supply were examined.

As nitrogen is one of the components to synthesize chlorophyll, which in turn involved in the photosynthesis that provide ATP for the nitrogen assimilation GOGAT cycle, measurement of chlorophyll content would reflect both the carbon assimilation and nitrogen assimilation process of the plants. Moreover, the balance of energy utilization and nitrogen assimilation would be an important factor for plant growth. Apart from chlorophyll, fresh weight was used as another parameter for measuring growth. In addition, since PII may alter regulation of nitrogen assimilation in plants that may indirectly altered the nitrogen storage or nitrogen allocation in seeds, the carbon to nitrogen ratio in seeds was measured to reflect the nitrogen status in seeds. Besides, as root length is an easily measurable growth parameter, it was chosen to indicate root growth.

3.3.1 Fresh weight of the young seedlings

The fresh weights of 10-day-old seedlings grown on MS agar plates were measured (Figure 3.4). The descriptive data (sample size, mean, and standard deviation) are

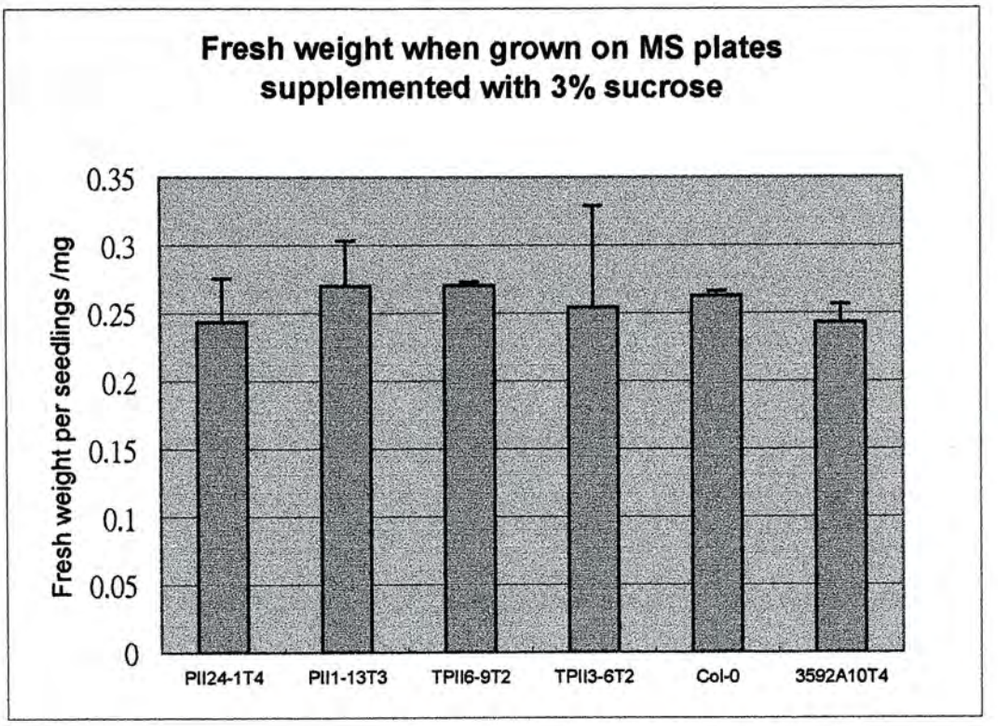


Figure 3.4: Fresh weight when grown on MS agar plates supplemented with 3% sucrose. Surface sterilized seeds were sown on MS agar plates supplemented with 3% sucrose and imbibed for 2 days in dark. The seedlings were allowed to grow for 10 days and the fresh weight of 10 seedlings of each line was measured as a single data point. Error bar represents the standard deviation of 2 data point. This experiment was repeated twice and similar results were obtained. Statistical analysis was shown in Table 3.1.

shown in Table 3.1A. The data was subjected to One-way ANOVA (α=0.937>0.05) (Table 3.1B). Statistic analysis indicated that there was no significant difference among the fresh weights of the samples. The significance level was set to 0.05.

3.3.2 Chlorophyll contents of shoots

The chlorophyll contents of the PII and truncated PII transgenic plants grown on MS agar plates supplemented with 3% sucrose were measured (Figure 3.5). The descriptive data (sample size, mean, and standard deviation) are shown in Table 3.2A. The data was subjected to One-way ANOVA (α=0.000<0.05) (Table 3.2B). Statistic analysis indicated that there were significant differences among the chlorophyll contents of the samples.

Since the variances of different populations showed to be equal (α=0.379>0.05) (Table 3.2C), Post Hoc Multiple Comparison using Bonferroni test was done and data from Col-0 was compared to different lines (Table 3.2D). The results indicated that one of the truncated lines, TPII3-6T2, had significantly lower chlorophyll contents than all other lines (α=0.000<0.05). The chlorophyll contents of all lines except TPII3-6T2 were not significantly different from Col-0 and TPII3-6T2 exhibited significantly lower chlorophyll contents than Col-0 (α=0.000<0.05).

A.

Descriptives

			N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
							Lower Bound	Upper Bound		
FW	PII transgenic plants	3592A10T3	2	.2431250	1.33E-02	9.37E-03	.1240044	.3622456	.23375	.25250
		Col-0	2	.2625000	3.54E-03	2.50E-03	.2307345	.2942655	.26000	.26500
		PII24-1T4	2	.2437500	3.18E-02	2.25E-02	-4.2E-02	.5296395	.22125	.26625
		PII1-13T3	2	.2700000	3.36E-02	2.38E-02	-3.2E-02	.5717723	.24625	.29375
		TPII6-9T2	2	.2706250	2.65E-03	1.88E-03	.2468009	.2944491	.26875	.27250
		TPII3-6T2	2	.2544643	7.50E-02	5.30E-02	-.4194181	.9283467	.20143	.30750
		Total	12	.2574107	2.94E-02	8.47E-03	.2387600	.2760614	.20143	.30750

B.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
FW	Between Groups	1.517E-03	5	3.034E-04	.229	.937
	Within Groups	7.962E-03	6	1.327E-03		
	Total	9.478E-03	11			

Table 3.1: Statistical analysis on data of Figure 3.4.

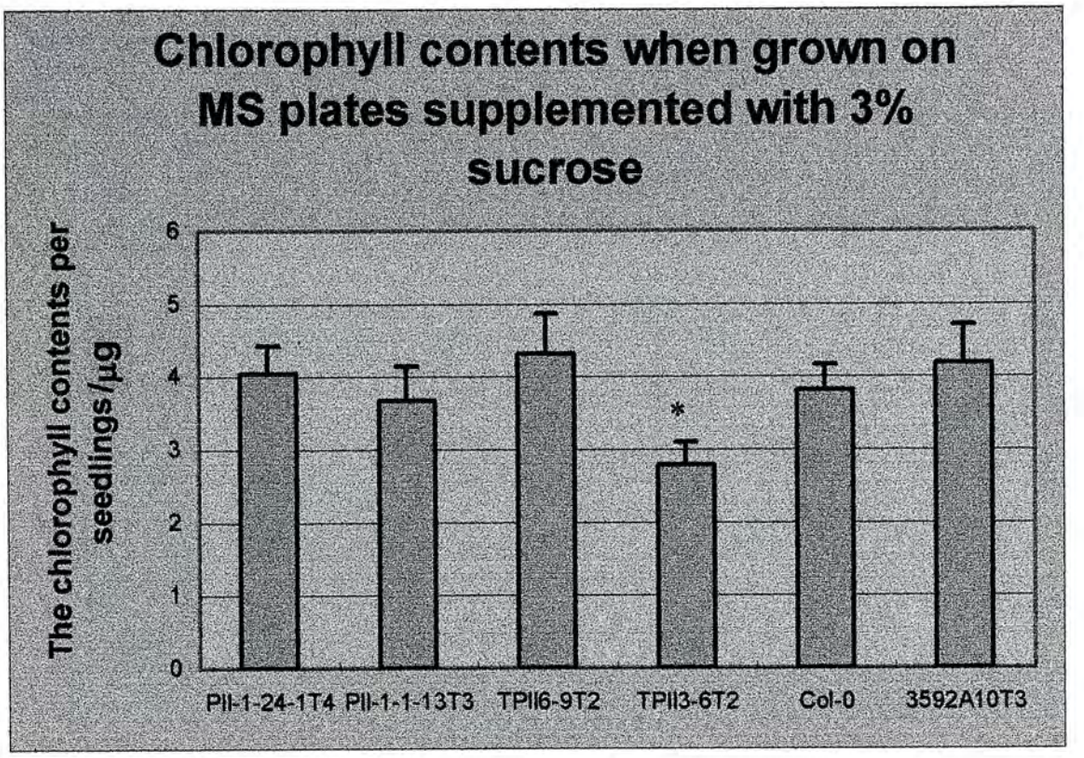


Figure 3.5: Chlorophyll contents when grown on MS agar plates supplemented with 3% sucrose. Surface sterilized seeds were sown on the plates, followed by an imbibition for 2 days in dark, and allowed to grow for 10 days. Ten seedlings of each line were pooled as one data point. Chlorophyll was measured using a spectrophotometry analysis as described in Materials and Methods. Error bar represents the standard deviation of 10 data points. '*' indicates a significant difference compared to the wild type Col-0. Statistical analysis was shown in Table 3.2.

A.

Descriptives

			N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
							Lower Bound	Upper Bound		
CHLOR2	TYPE2	Col-0(20mM)	10	3.807770	.359438	.113664	3.550644	4.064897	3.0115	4.2694
		359.2A10T3(20mM)	10	4.180408	.527106	.166686	3.803339	4.557477	3.3223	5.0957
		PII24-1T4(20mM)	10	4.047235	.378583	.119718	3.776414	4.318057	3.4636	4.6120
		PII1-13T3(20mM)	10	3.673938	.465035	.147057	3.341272	4.006604	3.0542	4.5253
		TPII6-9T2(20mM)	10	4.318330	.296524	9.38E-02	4.106210	4.530451	3.8997	4.7887
		TPII3-6T2(20mM)	10	2.784890	.314268	9.94E-02	2.560077	3.009704	2.2640	3.4055
		Total	60	3.802095	.634977	8.20E-02	3.638064	3.966127	2.2640	5.0957

B.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CHLOR2	Between Groups	15.209	5	3.042	19.144	.000
	Within Groups	8.580	54	.159		
	Total	23.789	59			

C.

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
CHLOR2	1.086	5	54	.379

D.

Multiple Comparisons

Dependent Variable: CHLOR2

Bonferroni

(I) TYPE2	(J) TYPE2	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Col-0(20mM)	359.2A10T3(20mM)	-.372638	.178	.620	-.920149	.174873
	PII24-1T4(20mM)	-.239465	.178	1.000	-.786976	.308046
	PII1-13T3(20mM)	.133832	.178	1.000	-.413679	.681344
	TPII6-9T2(20mM)	-.510560	.178	.089	-1.058071	3.70E-02
	TPII3-6T2(20mM)	1.022880*	.178	.000	.475369	1.570391
359.2A10T3(20mM)	Col-0(20mM)	.372638	.178	.620	-.174873	.920149
	PII24-1T4(20mM)	.133173	.178	1.000	-.414338	.680684
	PII1-13T3(20mM)	.506470	.178	.095	-4.1E-02	1.053982
	TPII6-9T2(20mM)	-.137922	.178	1.000	-.685433	.409589
	TPII3-6T2(20mM)	1.395518*	.178	.000	.848007	1.943029
PII24-1T4(20mM)	Col-0(20mM)	.239465	.178	1.000	-.308046	.786976
	359.2A10T3(20mM)	-.133173	.178	1.000	-.680684	.414338
	PII1-13T3(20mM)	.373297	.178	.614	-.174214	.920808
	TPII6-9T2(20mM)	-.271095	.178	1.000	-.818606	.276416
	TPII3-6T2(20mM)	1.262345*	.178	.000	.714834	1.809856
PII1-13T3(20mM)	Col-0(20mM)	-.133832	.178	1.000	-.681344	.413679
	359.2A10T3(20mM)	-.506470	.178	.095	-1.053982	4.10E-02
	PII24-1T4(20mM)	-.373297	.178	.614	-.920808	.174214
	TPII6-9T2(20mM)	-.644392*	.178	.010	-1.191904	-9.7E-02
	TPII3-6T2(20mM)	.889048*	.178	.000	.341536	1.436559
TPII6-9T2(20mM)	Col-0(20mM)	.510560	.178	.089	-3.7E-02	1.058071
	359.2A10T3(20mM)	.137922	.178	1.000	-.409589	.685433
	PII24-1T4(20mM)	.271095	.178	1.000	-.276416	.818606
	PII1-13T3(20mM)	.644392*	.178	.010	9.69E-02	1.191904
	TPII3-6T2(20mM)	1.533440*	.178	.000	.985929	2.080951
TPII3-6T2(20mM)	Col-0(20mM)	-1.022880*	.178	.000	-1.570391	-.475369
	359.2A10T3(20mM)	-1.395518*	.178	.000	-1.943029	-.848007
	PII24-1T4(20mM)	-1.262345*	.178	.000	-1.809856	-.714834
	PII1-13T3(20mM)	-.889048*	.178	.000	-1.436559	-.341536
	TPII6-9T2(20mM)	-1.533440*	.178	.000	-2.080951	-.985929

*. The mean difference is significant at the .05 level.

Table 3.2: Statistical analysis on data of Figure 3.5.

As the chlorophyll contents among the lines seemed to be different when grown on MS medium supplemented with 3% sucrose, the corresponding results of soil-grown plants were also monitored. Leaf discs of the same size, obtained from soil grown mature plants (by punching), were soaked in 800μl DMF in dark at 4°C overnight before subjected to spectrophotometric analysis (Figure 3.6). The descriptive data (sample size, mean, and standard deviation) were shown in Table 3.3A. The data were subjected to One-way ANOVA ($\alpha=0.025<0.05$) (Table 3.3B). The results suggest that there were significant differences among the chlorophyll contents of the samples. Since the variances of different populations were unequal ($\alpha=0.015<0.05$) (Table 3.3C), Post Hoc Multiple Comparison using Tamhane test was performed and Col-0 was compared to different lines. Statistical analysis showed that there was no significantly difference between the chlorophyll contents of Col-0 to any other lines. However, the test indicated that there was a significant difference between the chlorophyll contents of PII24-1T4 and TPII6-9T2. Such statistically difference was not analyzed further since it is more meaningful to perform the comparison between the wild type control, Col-0 and other lines.

In bacteria, PII protein functions in regulating ammonia assimilation which provides the main nitrogen source. Though ammonia is not the main nitrogen source for plants, it represents both a source of nitrogen and an environmental stress. The dosage of

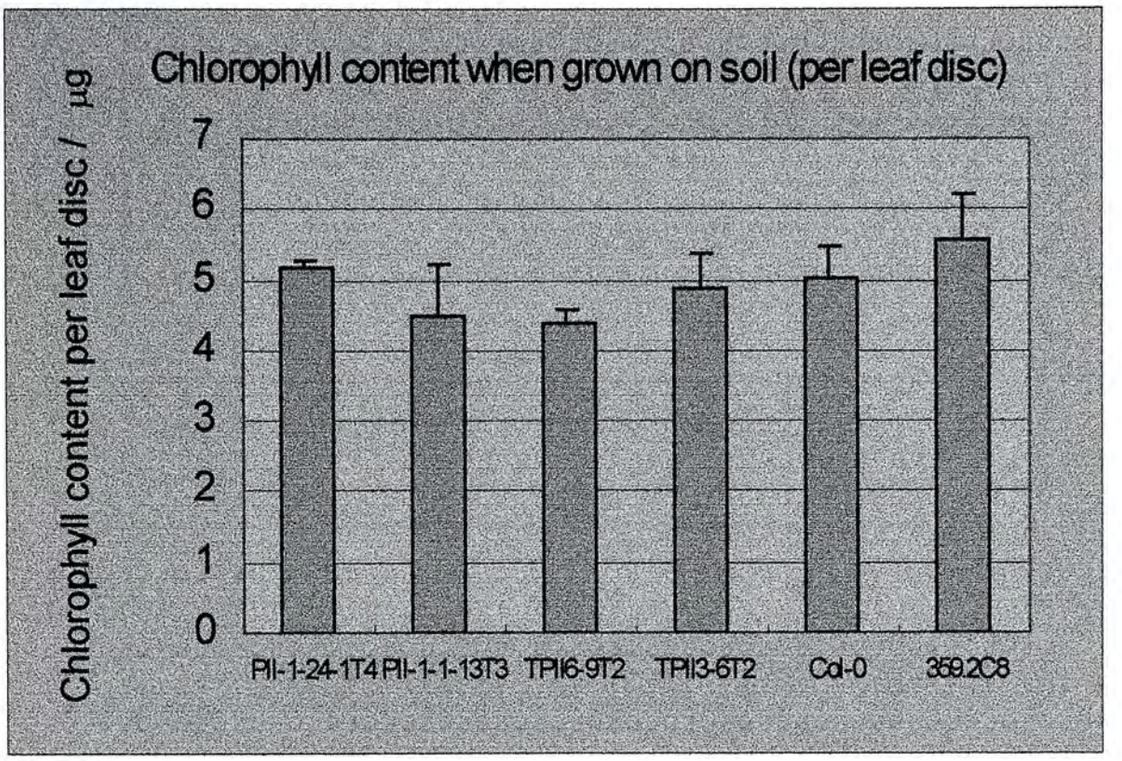


Figure 3.6: Chlorophyll contents when grown on soil (per leaf disc). Surface sterilized seeds were first sown and imbibed for 2 days in dark. They were then grown on MS agar plates supplemented with 3% sucrose for 14 days. Seedlings with similar size were chosen and transferred to soil for a further growth of 20 days. Five leave discs were collected from each line by punching and pooled as one data point. Error bar represents the standard deviation of four data points. The experiment was repeated twice and similar results were obtained. Statistical analysis was shown in Table 3.3.

A.

Descriptives

			N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
							Lower Bound	Upper Bound		
CHLOR	Construct	Col-0	4	5.039596	.43310136	.21655068	4.350445	5.728747	4.580750	5.553293
		359.2A10T4	4	5.585035	.63702843	.31851422	4.571396	6.598675	4.754787	6.091817
		PII24-1T4	4	5.188687	8.82E-02	4.41E-02	5.048351	5.329022	5.120835	5.307070
		PII1-13T3	4	4.490540	.73131401	.36565701	3.326873	5.654207	3.619007	5.290902
		TPII6-9T2	4	4.403455	.16140042	8.07E-02	4.146635	4.660276	4.186870	4.577335
		TPII3-6T2	4	4.903192	.46908901	.23454451	4.156778	5.649607	4.201887	5.189500
		Total	24	4.935084	.59204812	.12085132	4.685084	5.185084	3.619007	6.091817

B.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CHLOR	Between Groups	3.916	5	.783	3.400	.025
	Within Groups	4.146	18	.230		
	Total	8.062	23			

C.

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
CHLOR	3.861	5	18	.015

D.

Multiple Comparisons

Dependent Variable: CHLOR

Tamhane

(I) Construct	(J) Construct	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Col-0	359.2A10T4	-.5454396	.339	.972	-2.48400	1.393124
	PII24-1T4	-.1490908	.339	1.000	-1.85179	1.553609
	PII1-13T3	.54905583	.339	.988	-1.70797	2.806085
	TPII6-9T2	.63614042	.339	.565	-.863442	2.135723
	TPII3-6T2	.13640375	.339	1.000	-1.36107	1.633875
359.2A10T4	Col-0	.54543958	.339	.972	-1.39312	2.484003
	PII24-1T4	.39634875	.339	.996	-2.21031	3.003011
	PII1-13T3	1.094495	.339	.639	-1.19561	3.384596
	TPII6-9T2	1.181580	.339	.369	-1.23417	3.597331
	TPII3-6T2	.68184333	.339	.896	-1.25793	2.621614
PII24-1T4	Col-0	.14909083	.339	1.000	-1.55361	1.851790
	359.2A10T4	-.3963488	.339	.996	-3.00301	2.210314
	PII1-13T3	.69814667	.339	.915	-2.32115	3.717443
	TPII6-9T2	.78523125*	.339	.008	.27959805	1.290864
	TPII3-6T2	.28549458	.339	.996	-1.57815	2.149137
PII1-13T3	Col-0	-.5490558	.339	.988	-2.80608	1.707973
	359.2A10T4	-1.094495	.339	.639	-3.38460	1.195605
	PII24-1T4	-.6981467	.339	.915	-3.71744	2.321150
	TPII6-9T2	8.71E-02	.339	1.000	-2.75378	2.927951
	TPII3-6T2	-.4126521	.339	.999	-2.64672	1.821414
TPII6-9T2	Col-0	-.6361404	.339	.565	-2.13572	.86344175
	359.2A10T4	-1.181580	.339	.369	-3.59733	1.234171
	PII24-1T4	-.7852313*	.339	.008	-1.29086	-.279598
	PII1-13T3	-8.71E-02	.339	1.000	-2.92795	2.753782
	TPII3-6T2	-.4997367	.339	.853	-2.15888	1.159403
TPII3-6T2	Col-0	-.1364037	.339	1.000	-1.63387	1.361067
	359.2A10T4	-.6818433	.339	.896	-2.62161	1.257927
	PII24-1T4	-.2854946	.339	.996	-2.14914	1.578148
	PII1-13T3	.41265208	.339	.999	-1.82141	2.646718
	TPII6-9T2	.49973667	.339	.853	-1.15940	2.158876

*. The mean difference is significant at the .05 level.

Table 3.3: Statistical analysis on data of Figure 3.6.

ammonia in the medium is expected to significantly affect plant growth. Therefore, the effects of ammonia on shoot growth were tested, using chlorophyll contents as the measuring parameter, to examine the possible relationship between PII and ammonia metabolism.

The seeds of PII and truncated PII transgenic plants were sown, imbibed for 2 days in dark and grown on 3% sucrose ammonium-free MS agar plates supplemented with 0, 20, 100mM ammonium nitrate, respectively. Shoots of 10-day-old seedlings were used for chlorophyll content analysis as described in Materials and Methods.

All lines showed highest chlorophyll contents when grown on 3% sucrose MS medium supplemented with 20mM ammonium nitrate, comparing to that on 0 and 100mM ammonium nitrate (Figure 3.7). The descriptive data (sample size, mean, and standard deviation) were shown in Table 3.4A, 3.5A and 3.6A. The data were subjected to one-way ANOVA. Post Hoc Multiple Comparison using Bonferroni test was performed if the group variances were equal (Table 3.4C, $\alpha=0.074>0.05$) (Table 3.5C, $\alpha=0.379>0.05$). Post Hoc Multiple Comparison using Tamhane test was performed if the group variances were unequal (Table 3.6C, $\alpha=0.006<0.05$). Statistical analysis indicated that there is no significant difference in chlorophyll contents between the PII transgenic lines (PII24-1T4 and PII1-13T3) and the control plants (Col-0 and 359.2A10T3) in MS medium containing 3% sucrose

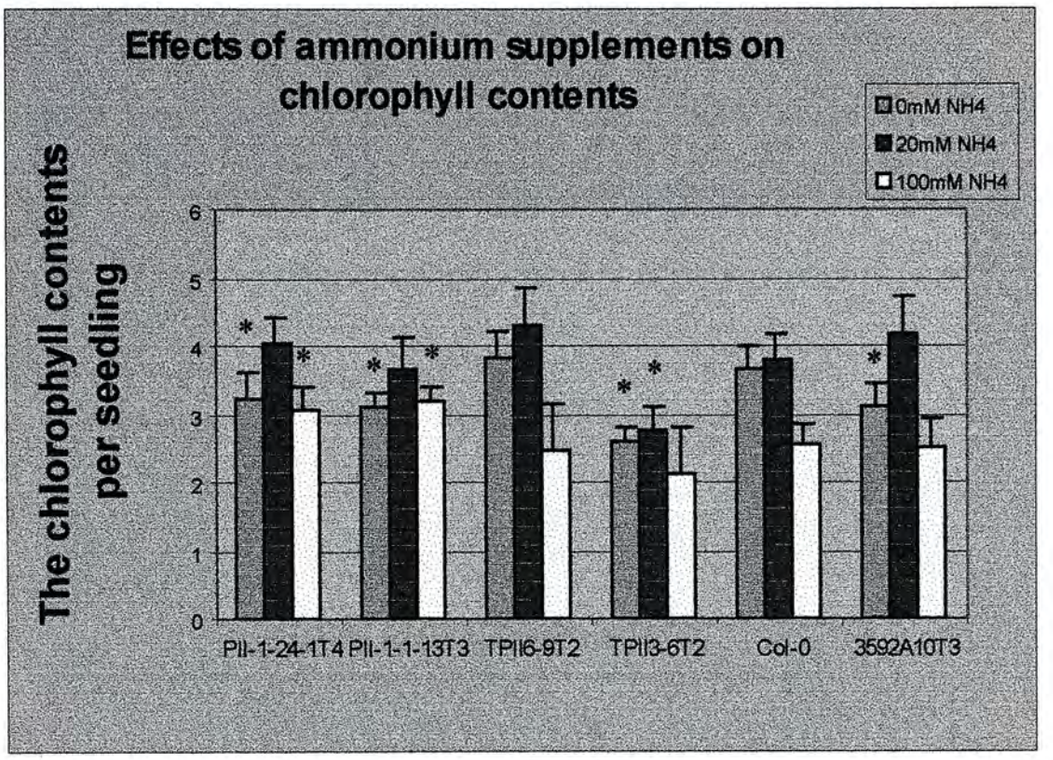


Figure 3.7: Effects of ammonium supplements on chlorophyll contents. Seeds of PII transgenic lines (PII24-1T4 and PII1-13T3), truncated PII transgenic lines (TPII6-9T2 and TPII3-6T2), wild type Col-0 and empty vector transformant control 359.2A10T3 were sown on ammonium-free MS agar plates supplemented with 3% sucrose. After imbibed in dark for 2 days, the seedlings were allowed to grow for 10 days under regular day-light cycle (16 hours light: 8 hours dark). Chlorophyll contents were measured using a spectrophometric method as described in Materials and Methods. '*' indicates that there is significant difference of the chlorophyll concentration of those lines when compared to Col-0 grown under the same ammonium nitrate concentration. Statistical analysis was shown in Table 3.4, 3.5 and 3.6.

A.

Descriptives

			N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
							Lower Bound	Upper Bound		
CHLOR1	TYPE1	Col-0(0mM)	10	3.641970	.364297	.115201	3.381368	3.902572	2.8433	3.9722
		359.2A10T3(0mM)	10	3.117624	.308862	9.77E-02	2.896677	3.338571	2.7924	3.7410
		PII24-1T4(0mM)	9	3.169723	.392273	.130758	2.868195	3.471250	2.5867	3.6826
		PII1-13T3(0mM)	10	3.103685	.209818	6.64E-02	2.953590	3.253779	2.8060	3.3703
		TPII6-9T2(0mM)	10	3.819156	.393079	.124302	3.537965	4.100348	3.4183	4.6409
		TPII3-6T2(0mM)	10	2.608099	.202099	6.39E-02	2.463527	2.752672	2.2028	2.8446
		Total	59	3.244624	.504807	6.57E-02	3.113071	3.376178	2.2028	4.6409

B.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CHLOR1	Between Groups	9.342	5	1.868	18.208	.000
	Within Groups	5.438	53	.103		
	Total	14.780	58			

C.

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
CHLOR1	1.773	5	53	.134

D.

Multiple Comparisons

Dependent Variable: CHLOR1

Bonferroni

(I) TYPE1	(J) TYPE1	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Col-0(0mM)	359.2A10T3(0mM)	.524346*	.143	.009	8.40E-02	.964726
	PII24-1T4(0mM)	.472247*	.147	.034	1.98E-02	.924695
	PII1-13T3(0mM)	.538285*	.143	.006	9.79E-02	.978665
	TPII6-9T2(0mM)	-.177187	.143	1.000	-.617567	.263194
	TPII3-6T2(0mM)	1.033870*	.143	.000	.593490	1.474251
359.2A10T3(0mM)	Col-0(0mM)	-.524346*	.143	.009	-.964726	-8.4E-02
	PII24-1T4(0mM)	-5.21E-02	.147	1.000	-.504547	.400349
	PII1-13T3(0mM)	1.394E-02	.143	1.000	-.426441	.454320
	TPII6-9T2(0mM)	-.701532*	.143	.000	-1.141913	-.261152
	TPII3-6T2(0mM)	.509525*	.143	.012	6.91E-02	.949905
PII24-1T4(0mM)	Col-0(0mM)	-.472247*	.147	.034	-.924695	-2.0E-02
	359.2A10T3(0mM)	5.210E-02	.147	1.000	-.400349	.504547
	PII1-13T3(0mM)	6.604E-02	.147	1.000	-.386410	.518486
	TPII6-9T2(0mM)	-.649434*	.147	.001	-1.101882	-.196986
	TPII3-6T2(0mM)	.561623*	.147	.005	.109176	1.014071
PII1-13T3(0mM)	Col-0(0mM)	-.538285*	.143	.006	-.978665	-9.8E-02
	359.2A10T3(0mM)	-1.39E-02	.143	1.000	-.454320	.426441
	PII24-1T4(0mM)	-6.60E-02	.147	1.000	-.518486	.386410
	TPII6-9T2(0mM)	-.715472*	.143	.000	-1.155852	-.275091
	TPII3-6T2(0mM)	.495586*	.143	.016	5.52E-02	.935966
TPII6-9T2(0mM)	Col-0(0mM)	.177187	.143	1.000	-.263194	.617567
	359.2A10T3(0mM)	.701532*	.143	.000	.261152	1.141913
	PII24-1T4(0mM)	.649434*	.147	.001	.196986	1.101882
	PII1-13T3(0mM)	.715472*	.143	.000	.275091	1.155852
	TPII3-6T2(0mM)	1.211057*	.143	.000	.770677	1.651438
TPII3-6T2(0mM)	Col-0(0mM)	-1.033870*	.143	.000	-1.474251	-.593490
	359.2A10T3(0mM)	-.509525*	.143	.012	-.949905	-6.9E-02
	PII24-1T4(0mM)	-.561623*	.147	.005	-1.014071	-.109176
	PII1-13T3(0mM)	-.495586*	.143	.016	-.935966	-5.5E-02
	TPII6-9T2(0mM)	-1.211057*	.143	.000	-1.651438	-.770677

*. The mean difference is significant at the .05 level.

Table 3.4: Statistical analysis on data of Figure 3.7 (0mM ammonium nitrate).

A.

Descriptives

			N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
							Lower Bound	Upper Bound		
CHLOR2	TYPE2	Col-0(20mM)	10	3.807770	.359438	.113664	3.550644	4.064897	3.0115	4.2694
		359.2A10T3(20mM)	10	4.180408	.527106	.166686	3.803339	4.557477	3.3223	5.0957
		PII24-1T4(20mM)	10	4.047235	.378583	.119718	3.776414	4.318057	3.4636	4.6120
		PII1-13T3(20mM)	10	3.673938	.465035	.147057	3.341272	4.006604	3.0542	4.5253
		TPII6-9T2(20mM)	10	4.318330	.296524	9.38E-02	4.106210	4.530451	3.8997	4.7887
		TPII3-6T2(20mM)	10	2.784890	.314268	9.94E-02	2.560077	3.009704	2.2640	3.4055
		Total	60	3.802095	.634977	8.20E-02	3.638064	3.966127	2.2640	5.0957

B.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CHLOR2	Between Groups	15.209	5	3.042	19.144	.000
	Within Groups	8.580	54	.159		
	Total	23.789	59			

C.

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
CHLOR2	1.086	5	54	.379

D.

Multiple Comparisons

Dependent Variable: CHLOR2

Bonferroni

(I) TYPE2	(J) TYPE2	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Col-0(20mM)	359.2A10T3(20mM)	-.372638	.178	.620	-.920149	.174873
	PII24-1T4(20mM)	-.239465	.178	1.000	-.786976	.308046
	PII1-13T3(20mM)	.133832	.178	1.000	-.413679	.681344
	TPII6-9T2(20mM)	-.510560	.178	.089	-1.058071	3.70E-02
	TPII3-6T2(20mM)	1.022880*	.178	.000	.475369	1.570391
359.2A10T3(20mM)	Col-0(20mM)	.372638	.178	.620	-.174873	.920149
	PII24-1T4(20mM)	.133173	.178	1.000	-.414338	.680684
	PII1-13T3(20mM)	.506470	.178	.095	-4.1E-02	1.053982
	TPII6-9T2(20mM)	-.137922	.178	1.000	-.685433	.409589
	TPII3-6T2(20mM)	1.395518*	.178	.000	.848007	1.943029
PII24-1T4(20mM)	Col-0(20mM)	.239465	.178	1.000	-.308046	.786976
	359.2A10T3(20mM)	-.133173	.178	1.000	-.680684	.414338
	PII1-13T3(20mM)	.373297	.178	.614	-.174214	.920808
	TPII6-9T2(20mM)	-.271095	.178	1.000	-.818606	.276416
	TPII3-6T2(20mM)	1.262345*	.178	.000	.714834	1.809856
PII1-13T3(20mM)	Col-0(20mM)	-.133832	.178	1.000	-.681344	.413679
	359.2A10T3(20mM)	-.506470	.178	.095	-1.053982	4.10E-02
	PII24-1T4(20mM)	-.373297	.178	.614	-.920808	.174214
	TPII6-9T2(20mM)	-.644392*	.178	.010	-1.191904	-9.7E-02
	TPII3-6T2(20mM)	.889048*	.178	.000	.341536	1.436559
TPII6-9T2(20mM)	Col-0(20mM)	.510560	.178	.089	-3.7E-02	1.058071
	359.2A10T3(20mM)	.137922	.178	1.000	-.409589	.685433
	PII24-1T4(20mM)	.271095	.178	1.000	-.276416	.818606
	PII1-13T3(20mM)	.644392*	.178	.010	9.69E-02	1.191904
	TPII3-6T2(20mM)	1.533440*	.178	.000	.985929	2.080951
TPII3-6T2(20mM)	Col-0(20mM)	-1.022880*	.178	.000	-1.570391	-.475369
	359.2A10T3(20mM)	-1.395518*	.178	.000	-1.943029	-.848007
	PII24-1T4(20mM)	-1.262345*	.178	.000	-1.809856	-.714834
	PII1-13T3(20mM)	-.889048*	.178	.000	-1.436559	-.341536
	TPII6-9T2(20mM)	-1.533440*	.178	.000	-2.080951	-.985929

*. The mean difference is significant at the .05 level.

Table 3.5: Statistical analysis on the data of Figure 3.7 (20mM ammonium nitrate).

A.

Descriptives

			N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
							Lower Bound	Upper Bound		
CHLOR3	TYPE3	Col-0(100mM)	9	2.5734	.2898	9.662E-02	2.3506	2.7962	2.23	3.05
		359.2A10T3(100mM)	9	2.5043	.4257	.1419	2.1771	2.8315	1.68	3.07
		PII24-1T4(100mM)	9	3.0749	.3121	.1040	2.8350	3.3148	2.66	3.49
		PII1-13T3(100mM)	9	3.2032	.2219	7.396E-02	3.0327	3.3738	3.01	3.57
		TPII6-9T2(100mM)	9	2.4594	.6976	.2325	1.9232	2.9956	1.30	3.29
		TPII3-6T2(100mM)	9	2.1439	.6503	.2168	1.6440	2.6438	1.00	2.60
		Total	54	2.6599	.5799	7.891E-02	2.5016	2.8181	1.00	3.57

B.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CHLOR3	Between Groups	7.250	5	1.450	6.584	.000
	Within Groups	10.572	48	.220		
	Total	17.822	53			

C.

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
CHLOR3	3.787	5	48	.006

D.

Multiple Comparisons

Dependent Variable: CHLOR3

Tamhane

(I) TYPE3	(J) TYPE3	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Col-0(100mM)	359.2A10T3(100mM)	6.912E-02	.221	1.000	-.5338	.6721
	PII24-1T4(100mM)	-.5015*	.221	.041	-.9894	-1.36E-02
	PII1-13T3(100mM)	-.6298*	.221	.002	-1.0524	-.2073
	TPII6-9T2(100mM)	.1140	.221	1.000	-.8286	1.0566
	TPII3-6T2(100mM)	.4295	.221	.786	-.4509	1.3099
359.2A10T3(100mM)	Col-0(100mM)	-6.91E-02	.221	1.000	-.6721	.5338
	PII24-1T4(100mM)	-.5706	.221	.081	-1.1840	4.274E-02
	PII1-13T3(100mM)	-.6990*	.221	.014	-1.2802	-.1177
	TPII6-9T2(100mM)	4.489E-02	.221	1.000	-.9244	1.0141
	TPII3-6T2(100mM)	.3604	.221	.955	-.5537	1.2744
PII24-1T4(100mM)	Col-0(100mM)	.5015*	.221	.041	1.363E-02	.9894
	359.2A10T3(100mM)	.5706	.221	.081	-4.27E-02	1.1840
	PII1-13T3(100mM)	-.1283	.221	.998	-.5747	.3180
	TPII6-9T2(100mM)	.6155	.221	.406	-.3290	1.5601
	TPII3-6T2(100mM)	.9310*	.221	.035	4.774E-02	1.8142
PII1-13T3(100mM)	Col-0(100mM)	.6298*	.221	.002	.2073	1.0524
	359.2A10T3(100mM)	.6990*	.221	.014	.1177	1.2802
	PII24-1T4(100mM)	.1283	.221	.998	-.3180	.5747
	TPII6-9T2(100mM)	.7438	.221	.176	-.1978	1.6855
	TPII3-6T2(100mM)	1.0593*	.221	.015	.1818	1.9368
TPII6-9T2(100mM)	Col-0(100mM)	-.1140	.221	1.000	-1.0566	.8286
	359.2A10T3(100mM)	-4.49E-02	.221	1.000	-1.0141	.9244
	PII24-1T4(100mM)	-.6155	.221	.406	-1.5601	.3290
	PII1-13T3(100mM)	-.7438	.221	.176	-1.6855	.1978
	TPII3-6T2(100mM)	.3155	.221	.998	-.7768	1.4078
TPII3-6T2(100mM)	Col-0(100mM)	-.4295	.221	.786	-1.3099	.4509
	359.2A10T3(100mM)	-.3604	.221	.955	-1.2744	.5537
	PII24-1T4(100mM)	-.9310*	.221	.035	-1.8142	-4.77E-02
	PII1-13T3(100mM)	-1.0593*	.221	.015	-1.9368	-.1818
	TPII6-9T2(100mM)	-.3155	.221	.998	-1.4078	.7768

*. The mean difference is significant at the .05 level.

Table 3.6: Statistical analysis on data of Figure 3.7 (100mM ammonium nitrate).

and 20mM ammonium nitrate (Table 3.6D, α=1.00>0.05). However, Post Hoc Multiple Comparisons using the Bonferroni test indicated that both of the PII transgenic lines contained significantly less chlorophyll than the control plants (Col-0 and 359.2A10T4) when grown on MS medium without any ammonium supplements (Table 3.4D). Such reductions of chlorophyll contents in the PII transgenic lines suggest a more severe growth retardation of PII transgenic lines under nitrogen starvation.

On MS medium containing 3% sucrose and 100mM ammonium nitrate, all lines seemed to be under stress and exhibited significantly lower chlorophyll contents (Figure 3.7). Post Hoc Multiple Comparisons using the Tamhane test indicated that both the PII transgenic lines, PII24-1T4 (Table 3.6D, α=0.041<0.05) and PII1-13T3 (Table 3.6D, α=0.002<0.05), contained significantly more chlorophyll than the control plants (Col-0 and 359.2A10T3) under such high ammonium stress. It seemed that PII transgenic lines may have higher tolerance towards ammonium stress. When grown on ammonium-free MS medium supplemented with 3% sucrose and 0, 20 and 100mM ammonium nitrate, truncated PII transgenic lines behaved very differently than PII transgenic lines. At both 0mM and 20mM ammonium nitrate, TPII3-6T2 contains significantly lower chlorophyll contents than Col-0 (Table 3.4D and Table 3.5D) while there was no significant difference in chlorophyll contents between

TPII6-9T2 and Col-0 at both growth conditions (Table 3.4D and Table 3.5D). Under high ammonium stress, both truncated lines showed no significant difference in the chlorophyll contents when compared to the controls, Col-0 and 359.2A10T3. (Table 3.6D)

3.3.3 Root lengths

Root length was chosen as a parameter to compare the growth of the transgenic plants and the control plants grown on normal MS medium. The results of root length measurement (Figure 3.8) were subjected to statistical analysis (Table 3.7).

The descriptive data (sample size, mean, and standard deviation) were summarized in Table 3.7A. Results of one-way ANOVA (α=0.000<0.05) (Table 3.7B) indicated that there were significant differences among root length of the samples. Since the variances of different populations were equal (α value=0.421>0.05) (Table 3.7C), Post Hoc Multiple Comparison using Bonferroni test was performed. When Col-0 was compared to different lines, statistical test suggested that all transgenic lines except 359.2A10T3 were not significantly different from Col-0 (Table 3.7D). Possible reason for the observed difference between the two controls, 359.2A10T3 and Col-0 was explained in the previous section.

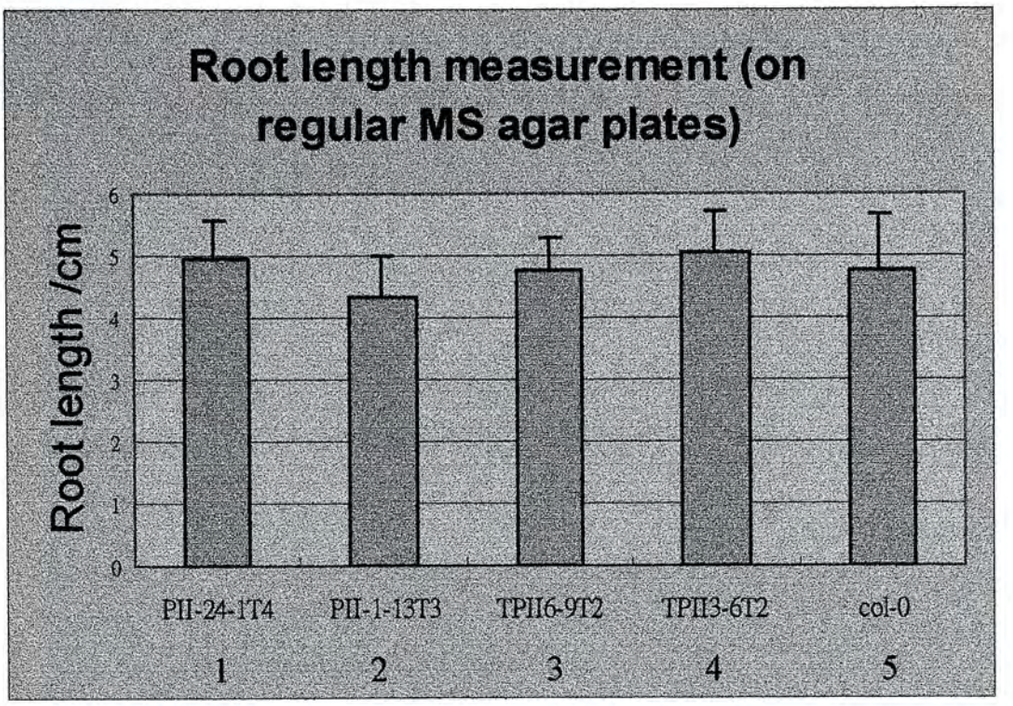


Figure 3.8: Root length measurement when the seedlings were grown on regular MS agar plates. Surface sterilized seedlings were sown onto regular MS agar plates supplemented with 3%sucrose, imbibed for 2 days in dark, before allowed to grow for another 10 days under a regular day-light cycle (16 hours light: 8 hours dark). Measurement of root length was performed on PII24-1T2 (lane 1), PII1-13T3 (lane 2), TPII6-9T2 (lane 3), TPII3-6T2 (lane 4) and the wild type parent Col-0 (lane 5). Root length of at least twenty-one samples for each line were measured. Error bar represents the standard deviation of the data points. The experiment was repeated twice and similar results were obtained. Statistical analysis was shown in Table 3.8.

A.

Descriptives

			N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
							Lower Bound	Upper Bound		
ROOT	0mM	PII24-1T4(0mM)	25	4.9720	.6202	.1240	4.7160	5.2280	3.50	5.80
		PII1-13T3(0mM)	21	4.3571	.6477	.1413	4.0623	4.6520	3.20	5.90
		TPII6-9T2(0mM)	24	4.7417	.5610	.1145	4.5048	4.9786	3.20	5.70
		TPII3-6T2(0mM)	22	5.0545	.6566	.1400	4.7634	5.3457	3.70	6.00
		Col-0(0mM)	22	4.7682	.9052	.1930	4.3668	5.1695	2.00	5.80
		359.2A10T3(0mM)	26	3.7500	.6389	.1253	3.4919	4.0081	1.70	4.70
		Total	140	4.5943	.8067	6.818E-02	4.4595	4.7291	1.70	6.00

B.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
ROOT	Between Groups	29.128	5	5.826	12.729	.000
	Within Groups	61.327	134	.458		
	Total	90.455	139			

C.

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
ROOT	1.000	5	134	.421

D.

Multiple Comparisons

Dependent Variable: ROOT

Bonferroni

(I) 0mM	(J) 0mM	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
PII24-1T4(0mM)	PII1-13T3(0mM)	.6149*	.200	.039	1.636E-02	1.2134
	TPII6-9T2(0mM)	.2303	.193	1.000	-.3475	.8081
	TPII3-6T2(0mM)	-8.25E-02	.198	1.000	-.6736	.5085
	Col-0(0mM)	.2038	.198	1.000	-.3872	.7949
	359.2A10T3(0mM)	1.2220*	.189	.000	.6556	1.7884
PII1-13T3(0mM)	PII24-1T4(0mM)	-.6149*	.200	.039	-1.2134	-1.64E-02
	TPII6-9T2(0mM)	-.3845	.202	.889	-.9887	.2196
	TPII3-6T2(0mM)	-.6974*	.206	.014	-1.3142	-8.06E-02
	Col-0(0mM)	-.4110	.206	.727	-1.0279	.2058
	359.2A10T3(0mM)	.6071*	.198	.040	1.392E-02	1.2004
TPII6-9T2(0mM)	PII24-1T4(0mM)	-.2303	.193	1.000	-.8081	.3475
	PII1-13T3(0mM)	.3845	.202	.889	-.2196	.9887
	TPII3-6T2(0mM)	-.3129	.200	1.000	-.9097	.2839
	Col-0(0mM)	-2.65E-02	.200	1.000	-.6233	.5703
	359.2A10T3(0mM)	.9917*	.191	.000	.4193	1.5640
TPII3-6T2(0mM)	PII24-1T4(0mM)	8.255E-02	.198	1.000	-.5085	.6736
	PII1-13T3(0mM)	.6974*	.206	.014	8.056E-02	1.3142
	TPII6-9T2(0mM)	.3129	.200	1.000	-.2839	.9097
	Col-0(0mM)	.2864	.204	1.000	-.3233	.8960
	359.2A10T3(0mM)	1.3045*	.196	.000	.7188	1.8903
Col-0(0mM)	PII24-1T4(0mM)	-.2038	.198	1.000	-.7949	.3872
	PII1-13T3(0mM)	.4110	.206	.727	-.2058	1.0279
	TPII6-9T2(0mM)	2.652E-02	.200	1.000	-.5703	.6233
	TPII3-6T2(0mM)	-.2864	.204	1.000	-.8960	.3233
	359.2A10T3(0mM)	1.0182*	.196	.000	.4325	1.6039
359.2A10T3(0mM)	PII24-1T4(0mM)	-1.2220*	.189	.000	-1.7884	-.6556
	PII1-13T3(0mM)	-.6071*	.198	.040	-1.2004	-1.39E-02
	TPII6-9T2(0mM)	-.9917*	.191	.000	-1.5640	-.4193
	TPII3-6T2(0mM)	-1.3045*	.196	.000	-1.8903	-.7188
	Col-0(0mM)	-1.0182*	.196	.000	-1.6039	-.4325

*. The mean difference is significant at the .05 level.

Table 3.7: Statistical analysis on the data in Figure 3.8.

To test whether there was a differential response towards nitrogen starvation, similar root length measurements were performed on plant samples grown on nitrogen-free MS agar medium. As expected, the average root length of wild-type Col-0 was shorter than that grown on regular MS medium which contains sufficient exogenous nitrogen supply (Figure 3.8 and 3.9). Reduction of root length under nitrogen-free medium was also observed in the PII transgenic lines, PII24-1T4 (Figure 3.8, lane 1 & Figure 3.9, lane 1) & PII1-13T3 (Figure 3.8, lane 2 & Figure 3.9, lane 2) and the truncated PII transgenic lines, TPII6-9T2 (Figure 3.8, lane 3 & Figure 3.9, lane 3) & TPII3-6T2 (Figure 3.8, lane 4 & Figure 3.9, lane 4).

The root lengths of all lines grown on nitrogen-free MS medium were subjected to statistic analysis and results were summarized in Table 3.8A-D. The descriptive data (sample size, mean, and standard deviation) were shown in Table 3.8A. The results of one-way ANOVA (α value=0.000<0.05) (Table 3.8B) indicated that there were significant differences among root length of the samples. Since the variances of different populations were equal (α value=0.064>0.05) (Table3.8C), Post Hoc Multiple Comparison using Bonferroni test was performed and Col-0 was compared to different lines. No significant difference was found in the root length when comparing either of the PII overexpressing lines to Col-0, when grown on the nitrogen-free MS medium. However, both truncated PII transgenic lines, TPII6-9T2

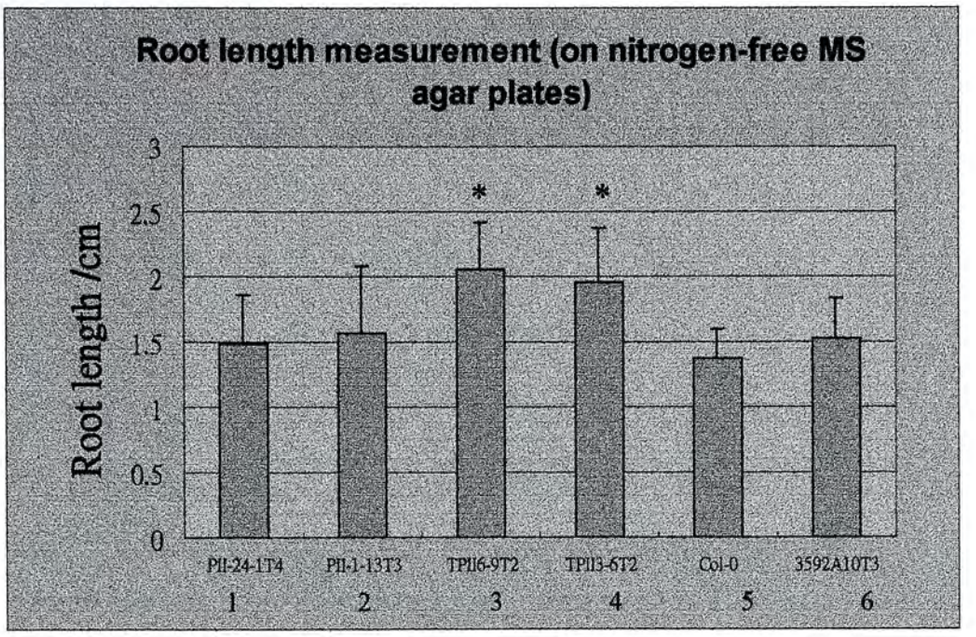


Figure 3.9: Root length measurement when the seedlings were grown on nitrogen-free MS agar plates. Surface sterilized seeds were sown on nitrogen-free MS agar plates supplemented with 3% sucrose, imbibed for 2 days in dark, before allowed to grow for another 10 days under a regular day-light cycle (16 hours light: 8 hours dark). Potassium ion was added in form of potassium chloride so that the final potassium ion concentration was equivalent to that of the regular MS medium. Measurement of root length was performed on PII24-1T2 (lane 1), PII1-13T3 (lane 2), TPII6-9T2 (lane 3), TPII3-6T2 (lane 4), wild type Col-0 (lane 5) and the empty vector transformant control 359.2A10T3 (lane 6). Root lengths of at least nineteen samples for each line were measured. Error bar represents standard deviations of the data points. The experiment was repeated twice and similar results were obtained. '*' indicates a significant difference compared to the wild type Col-0. Statistical analysis was shown in Table 3.8.

Descriptives

			N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
							Lower Bound	Upper Bound		
ROOT	constructs(ms+KCl)	col-0	19	1.3737	.2281	5.234E-02	1.2637	1.4836	1.00	1.80
		TPII3-6T2	45	1.9622	.4097	6.108E-02	1.8391	2.0853	1.30	3.10
		TPII6-9T2	48	2.0583	.3584	5.173E-02	1.9543	2.1624	1.10	2.70
		PII-1-13T3	23	1.5739	.5047	.1052	1.3557	1.7922	.80	2.80
		PII-24-1T4	39	1.4923	.3702	5.928E-02	1.3723	1.6123	.90	2.50
		3592A10T3	52	1.5231	.3129	4.339E-02	1.4360	1.6102	.80	2.30
		Total	226	1.7115	.4477	2.978E-02	1.6528	1.7702	.80	3.10

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
ROOT	2.111	5	220	.065

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
ROOT	Between Groups	14.926	5	2.985	21.773	.000
	Within Groups	30.164	220	.137		
	Total	45.090	225			

Multiple Comparisons

Dependent Variable: ROOT

Bonferroni

(I) constructs(ms+KCl)	(J) constructs(ms+KCl)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
col-0	TPII3-6T2	-.5885*	.101	.000	-.8892	-.2879
	TPII6-9T2	-.6846*	.100	.000	-.9825	-.3868
	PII-1-13T3	-.2002	.115	1.000	-.5409	.1404
	PII-24-1T4	-.1186	.104	1.000	-.4260	.1888
	3592A10T3	-.1494	.099	1.000	-.4440	.1452
TPII3-6T2	col-0	.5885*	.101	.000	.2879	.8892
	TPII6-9T2	-9.61E-02	.077	1.000	-.3241	.1319
	PII-1-13T3	.3883*	.095	.001	.1067	.6700
	PII-24-1T4	.4699*	.081	.000	.2295	.7103
	3592A10T3	.4391*	.075	.000	.2154	.6629
TPII6-9T2	col-0	.6846*	.100	.000	.3868	.9825
	TPII3-6T2	9.611E-02	.077	1.000	-.1319	.3241
	PII-1-13T3	.4844*	.094	.000	.2058	.7631
	PII-24-1T4	.5660*	.080	.000	.3291	.8029
	3592A10T3	.5353*	.074	.000	.3153	.7552
PII-1-13T3	col-0	.2002	.115	1.000	-.1404	.5409
	TPII3-6T2	-.3883*	.095	.001	-.6700	-.1067
	TPII6-9T2	-.4844*	.094	.000	-.7631	-.2058
	PII-24-1T4	8.161E-02	.097	1.000	-.2073	.3705
	3592A10T3	5.084E-02	.093	1.000	-.2243	.3260
PII-24-1T4	col-0	.1186	.104	1.000	-.1888	.4260
	TPII3-6T2	-.4699*	.081	.000	-.7103	-.2295
	TPII6-9T2	-.5660*	.080	.000	-.8029	-.3291
	PII-1-13T3	8.16E-02	.097	1.000	-.3705	.2073
	3592A10T3	-3.08E-02	.078	1.000	-.2635	.2020
3592A10T3	col-0	.1494	.099	1.000	-.1452	.4440
	TPII3-6T2	-.4391*	.075	.000	-.6629	-.2154
	TPII6-9T2	-.5353*	.074	.000	-.7552	-.3153
	PII-1-13T3	-5.08E-02	.093	1.000	-.3260	.2243
	PII-24-1T4	3.077E-02	.078	1.000	-.2020	.2635

*. The mean difference is significant at the .05 level.

Table 3.8: Statistical analysis on data in Figure 3.9.

(Figure 3.9, lane 3) and TPII3-6T2 (Figure 3.9, lane 4) showed significantly longer root length than the wild-type control Col-0 (Figure 3.9, lane 5), the control empty vector transformant 359.2A10T3 (Figure 3.9, lane 6) as well as the two PII transgenic lines (Figure 3.9, lanes 1 & 2) (Table 3.8D).

3.3.4 Carbon and nitrogen status of seeds

To test if there was any effect of PII on the nitrogen content of seeds (ultimate storage organ), total percentage of nitrogen and the nitrogen to carbon ratio were determined (Figure 3.10 A & B).

It was found that both truncated lines have higher nitrogen to carbon ratio (Figure 3.10A, lanes 3 & 4) and a higher percentage of nitrogen in seeds than other lines. (Figure 3.10B, lanes 3 & 4). On the other hand, PII transgenic lines did not exhibit any difference from Col-0 and the empty vector transformant control, 359.2A10T3 (Figure 3.10A, lanes 1 & 2, Figure 3.10B, lanes 1 & 2).

However, statistical analysis using ANOVA dos not support that the truncated lines are significant difference from other lines. It may due to the fact that only three samples from each line were tested and such sample sizes are small.

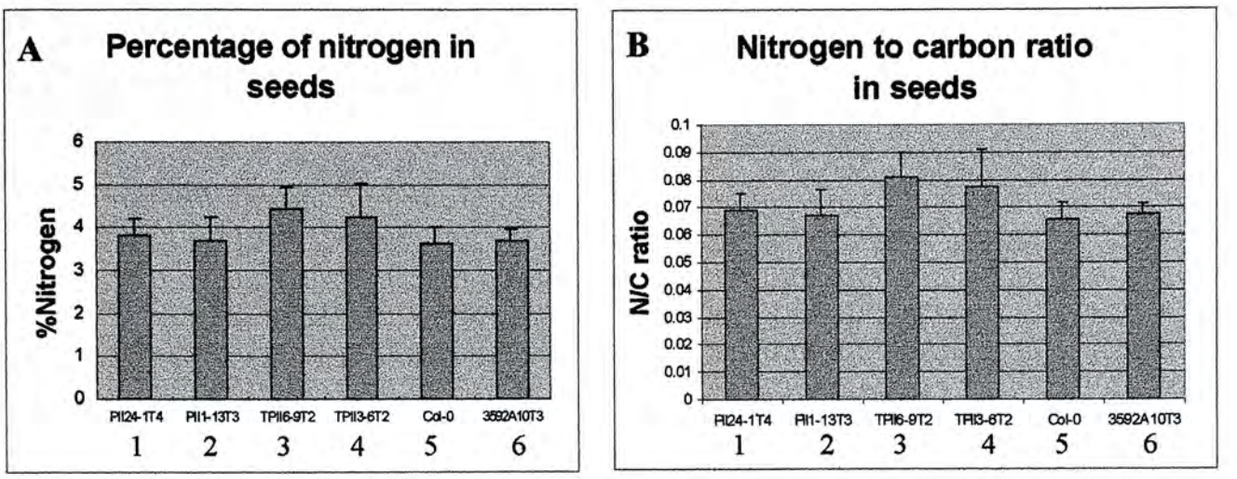


Figure 3.10: Measurement of percentage of nitrogen (A) and nitrogen to carbon ratio (B) in seeds. Seeds were first sown on regular MS agar plates supplemented with 3% sucrose, imbibed for 2 days in dark and allowed to grow for 11 days before transferred to soil as describe in Methods and Materials. All plants were allowed to grow until brown dry seeds were obtained. All lines were grown on the same tray with similar water and light supply and no additional fertilizers were added. After collecting the seeds, one hundred seeds of each of the PII transgenic lines PII24-1T4 (lane 1), PII1-13T3 (lane2), truncated PII transgenic lines TPII6-9T2 (lane 3), TPII3-6T2 (lane 4), wild-type parent Col-0 (lane 5) and empty vector transformant control (lane 6) were used for the measurement. Percentage of nitrogen and the N/C ratio were analyzed with a CHN/S analyzer. Each error bar represents the standard deviation of the result of duplicated samples. The experiment was repeated twice and consistent results were obtained.

3.4 Expression of nitrogen assimilatory genes in PII and truncated PII transgenic lines

The expression levels of nitrogen assimilatory genes were measured in the PII and truncated PII transgenic plants. The hypothesis is that overexpressing PII or truncated PII may alter the internal C/N ratio which may directly or indirectly affect the expression of related genes. Northern blot analyses were performed and the relative gene expression levels were estimated. Surface-sterilized seeds were first sown on regular MS agar plates and grown for 11 days before transferred to soil for a further growth for 14 days. Full rosette leaves were harvested after 48 hours of continuous light or dark treatment for RNA extraction.

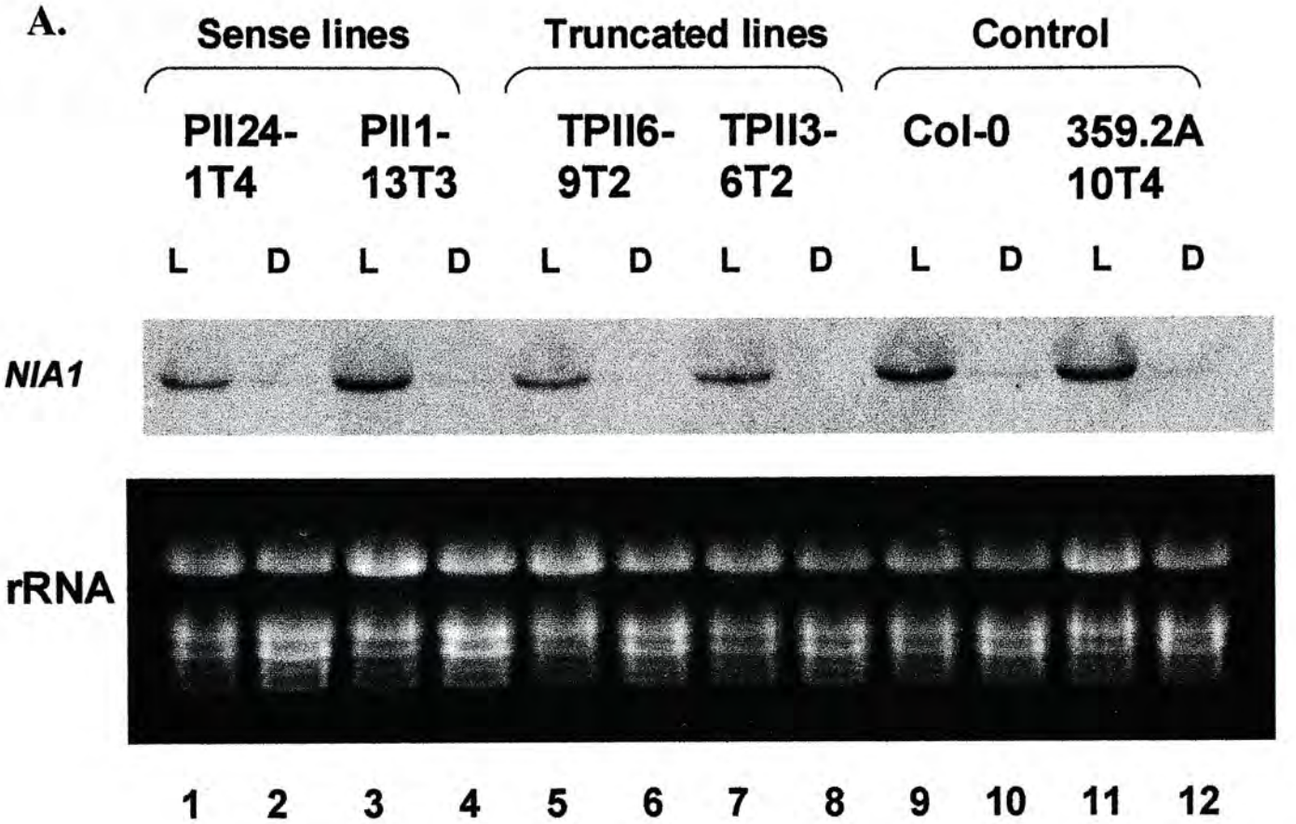
In some experiments, surface-sterilized seeds were first sown onto regular MS agar plates, grown for 14 days, before transferred to new regular MS agar plates containing supplements as specified (see Materials and Methods).

3.4.1 Nitrate reductase

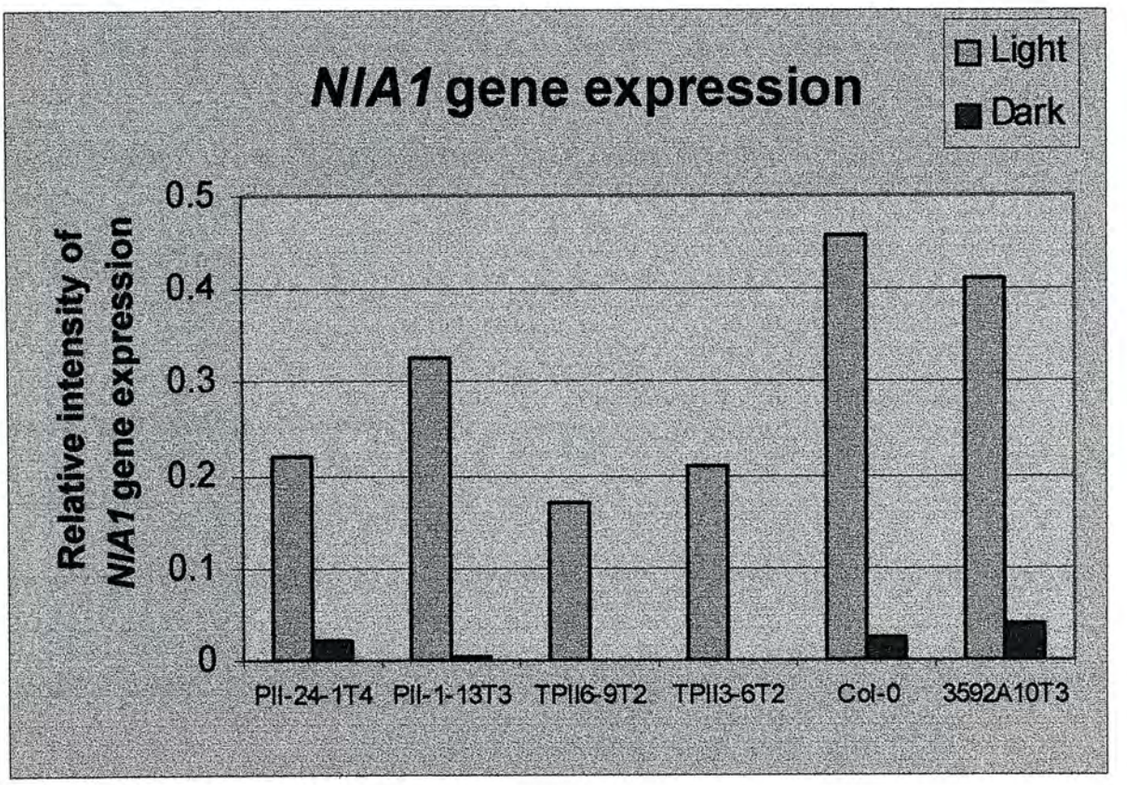
The *NIA1* gene, encoding for a nitrate reductase in *Arabidopsis thaliana*, is induced by light and repressed by dark treatment (Figure 3.11A). Only low level of *NIA1* expression was observed in dark. In the light, all PII and truncated PII transgenic

Figure 3.11: Steady state levels of *NIA1* mRNA. (A) Results of Northern Blot analysis; (B) Intensity of *NIA1* hybridization signal normalized to rRNA signal on ethidium bromide gel. PII transgenic lines PII24-1T4 (lanes 1 and 2) and PII1-13T3 (lanes 3 and 4), truncated PII transgenic lines TPII6-9T2 (lanes 5 and 6) and TPII3-6T2 (lanes 7 and 8), wild type parent Col-0 (lanes 9 and 10), and the control empty vector transformant 359.2A10T4 (lanes 11 and 12) were grown on regular MS agar plates under a regular day-night cycle for 14 days before subjected to continuous light (lanes 1, 3, 5, 7, 9 and 11) or continuous dark (lanes, 2, 4, 6, 8, 10, 12) treatment for 48 hours. Total leaf RNA was extracted and an aliquot of 15μg from each line was loaded on the gel. DIG-labeled probe was used to detect *NIA1* mRNA. L: light-grown plants; D: dark-adapted plants. Numerical values of the intensity of *NIA1* hybridization signal normalized to rRNA signal on ethidium bromide gel were shown on Table 3.11.

A.


Sense lines
Truncated lines
Control
PII24-1T4
PII1-13T3
TPII6-9T2
TPII3-6T2
Col-0
359.2A 10T4
L D L D L D L D L D L D
NIA1
rRNA
1 2 3 4 5 6 7 8 9 10 11 12

B.


NIA1 gene expression
Light
Dark
Relative intensity of NIA1 gene expression
0.5
0.4
0.3
0.2
0.1
0
PII-24-1T4
PII-1-13T3
TPII6-9T2
TPII3-6T2
Col-0
3592A10T3

lines showed lower levels of *NIA1* gene expression when compared to the control plants (Col-0 & 3592A10T3). Effects were more obvious in the two truncated lines (Figure 3.11A, lanes 5 and 7; Figure 3.11B, lanes 5-8). Relative Northern blot signals were normalized to rRNA quantity using quantitation program of the Gel Doc scanning machine (Table 3.9).

The other nitrate reductase gene, *NIA2*, was also induced by light and strongly repressed by the dark treatment (Figure 3.12A). In the light, all PII and truncated PII transgenic lines showed lower level of NIA2 gene expression when compared to the control plants (Col-0 & 359.2A10T3). This effect was more obvious in the truncated lines, TPII3-6T2 (Figure 3.12A, lane 7; Figure 3.12B, lane 7). Relative Northern blot signals were normalized to rRNA quantity using quantitation program of the Gel Doc scanning machine (Table 3.10).

3.4.2 Glutamine synthetase

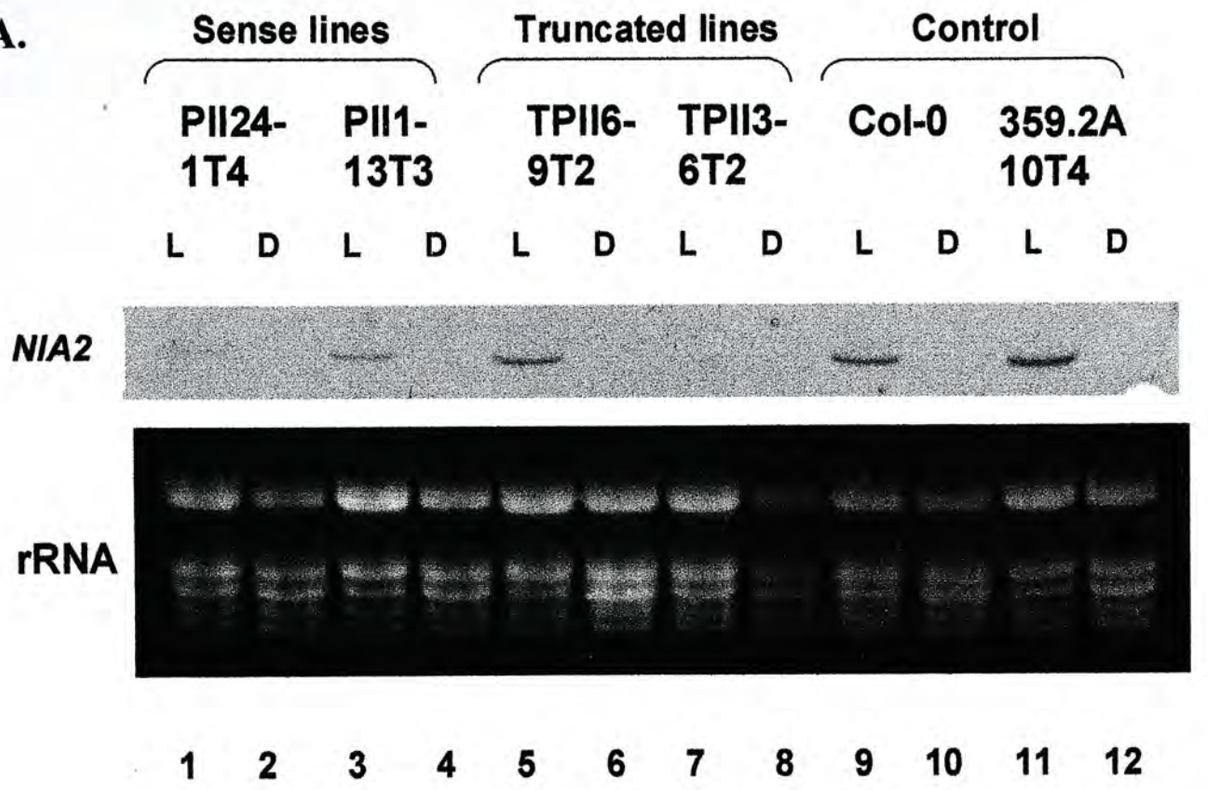
The chloroplastic GS gene (*GSL1*) expression was high in light but disminished under the dark treatment (Figure 3.13A). After 48 hours in the light, both truncated PII transgenic lines exhibited elevated levels of *GSL1* gene expression compared to Col-0 (Figure 3.13A, lanes 5 and 7) whereas both PII transgenic lines showed similar expression level to that of the control plants (Figure 3.13A, lanes 1 and 3). The

Table 3.9: Relative quantitation of the expression of *NIA1* gene.

Constructs	Growth condition	*NIA1* signal intensity	28s RNA signal intensity	Relative intensity (X1000)	Fold changes
PII24-1T4	Light	1.90	8551	0.22	0.48
PII24-1T4	Dark	0.20	9091	0.02	0.05
PII1-13T3	Light	3.70	11326	0.33	0.72
PII1-13T3	Dark	0.05	9871	0.01	0.01
TPII6-9T2	Light	1.70	10235	0.17	0.37
TPII6-9T2	Dark	0.00	8316	0.00	0.00
TPII3-6T2	Light	1.80	8499	0.21	0.46
TPII3-6T2	Dark	0.00	6990	0.00	0.00
Col-0	Light	3.70	8128	0.46	1.00
Col-0	Dark	0.17	7170	0.02	0.05
359.2A10T3	Light	4.20	10300	0.41	0.90
359.2A10T3	Dark	0.34	9094	0.04	0.08

Figure 3.12: Steady state levels of *NIA2* mRNA. (A) Results of Northern Blot analysis; (B) Intensity of *NIA2* hybridization signal normalized to rRNA signal on ethidium bromide gels. PII transgenic lines PII24-1T4 (lanes 1 and 2) and PII1-13T3 (lanes 3 and 4), truncated PII transgenic lines TPII6-9T2 (lanes 5 and 6) and TPII3-6T2 (lanes 7 and 8), wild type parent Col-0 (lanes 9 and 10), and the control empty vector transformant 359.2A10T4 (lanes 11 and 12) were grown on regular MS agar plates supplemented with 3% sucrose, imbibed in dark for 2 days, and subsequently grown under a regular day-night cycle for 14 days before subjected to continuous light (lanes 1, 3, 5, 7, 9 and 11) or continuous dark (lanes, 2, 4, 6, 8, 10, 12) treatment for 48 hours. Total leaf RNA was extracted and an aliquot of 15μg from each line was loaded onto the gel. DIG-labeled probes were used to detect *NIA2* mRNA. L: light-grown plants; D: dark-adapted plants. Numerical values of intensity of *NIA2* hybridization signal normalized to rRNA signal on ethidium bromide gel were shown on Table 3.10.

A.


Sense lines
Truncated lines
Control
PII24-1T4
PII1-13T3
TPII6-9T2
TPII3-6T2
Col-0
359.2A 10T4
L D L D L D L D L D L D
NIA2
rRNA
1 2 3 4 5 6 7 8 9 10 11 12

B.

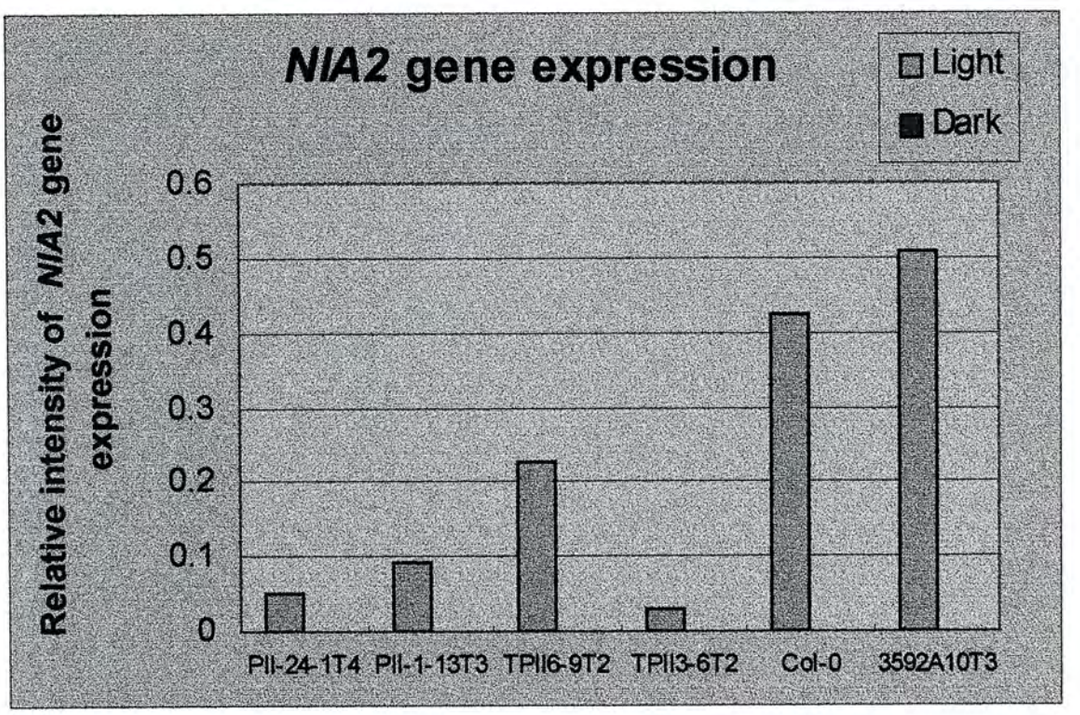
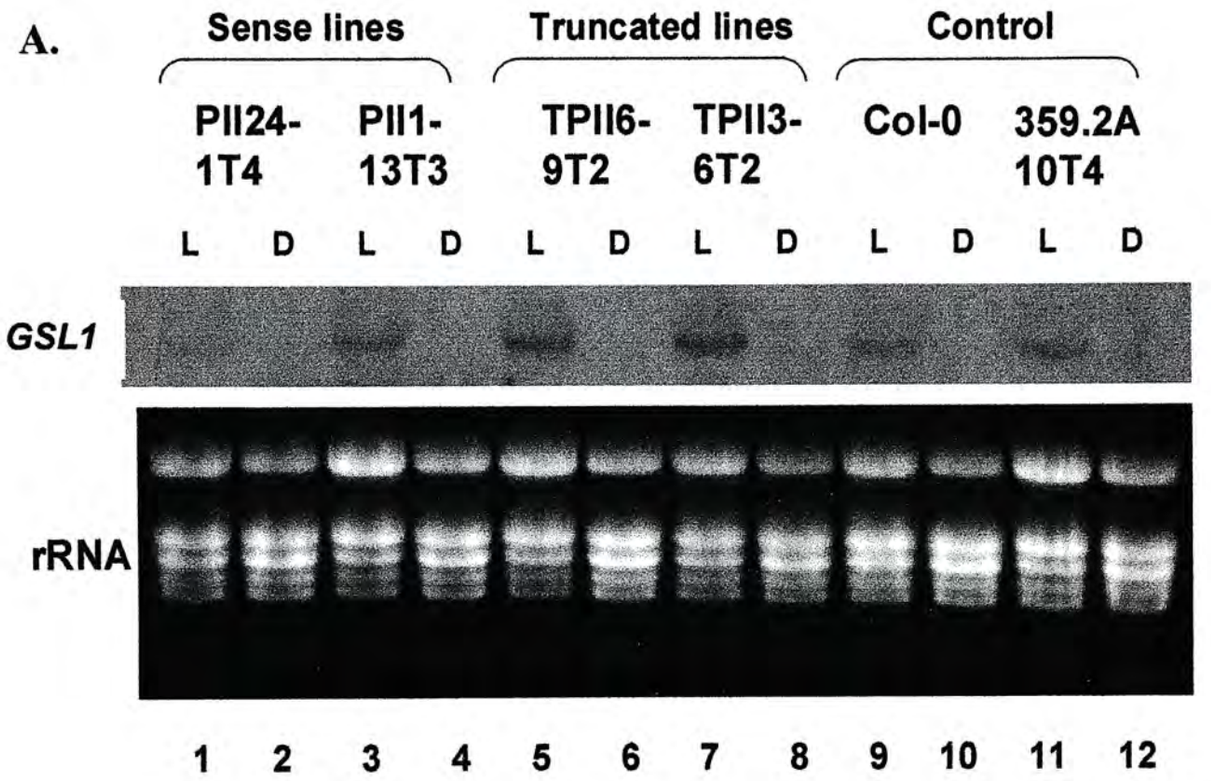

NIA2 gene expression
Light
Dark
Relative intensity of NIA2 gene expression
0.6
0.5
0.4
0.3
0.2
0.1
0
PII-24-1T4
PII-1-13T3
TPII6-9T2
TPII3-6T2
Col-0
3592A10T3

Table 3.10: Relative quantitation of the expression of *NIA2* gene

Constructs	Growth condition	*NIA 2* signal intensity	28s RNA signal intensity	Relative intensity (X1000)	Fold changes
PII24-1T4	Light	0.01	10342	0.05	0.11
PII24-1T4	Dark	0.00	6832	0.00	0.00
PII1-13T3	Light	0.01	11738	0.09	0.22
PII1-13T3	Dark	0.00	8761	0.00	0.00
TPII6-9T2	Light	0.02	10660	0.23	0.53
TPII6-9T2	Dark	0.00	9245	0.00	0.00
TPII3-6T2	Light	0.00	10534	0.03	0.07
TPII3-6T2	Dark	0.00	3918	0.00	0.00
Col-0	Light	0.03	7339	0.42	1.00
Col-0	Dark	0.00	5827	0.00	0.00
359.2A10T3	Light	0.05	9238	0.51	1.20
359.2A10T3	Dark	0.00	8512	0.00	0.00

Figure 3.13: Steady state levels of *GSL1* mRNA. (A) Results of Northern Blot analysis; (B) Intensity of *GSL1* hybridization signal normalized to rRNA signal on ethidium bromide gels. PII transgenic lines PII24-1T4 (lanes 1 and 2) and PII1-13T3 (lanes 3 and 4), truncated PII transgenic lines TPII6-9T2 (lanes 5 and 6) and TPII3-6T2 (lanes 7 and 8), wild type parent Col-0 (lanes 9 and 10), and the control empty vector transformant 359.2A10T4 (lanes 11 and 12) were grown on regular MS agar plates, imbibed in dark for 2 days, and subsequently grown under a regular day-night cycle for 14 days before subjected to continuous light (lanes 1, 3, 5, 7, 9 and 11) or continuous dark (lanes, 2, 4, 6, 8, 10, 12) treatment for 48 hours. Total leaf RNA was extracted and an aliquot of 15μg from each line was loaded onto the gel. DIG-labeled probes were used to detect *GSL1* mRNA. L: light-grown plants; D: dark-adapted plants. Numerical values of intensity of *GSL1* hybridization signal normalized to rRNA signal on ethidium bromide gel were shown on Table 3.11.

A.


Sense lines
Truncated lines
Control
PII24-1T4
PII1-13T3
TPII6-9T2
TPII3-6T2
Col-0
359.2A 10T4
L D L D L D L D L D L D
GSL1
rRNA
1 2 3 4 5 6 7 8 9 10 11 12

B.

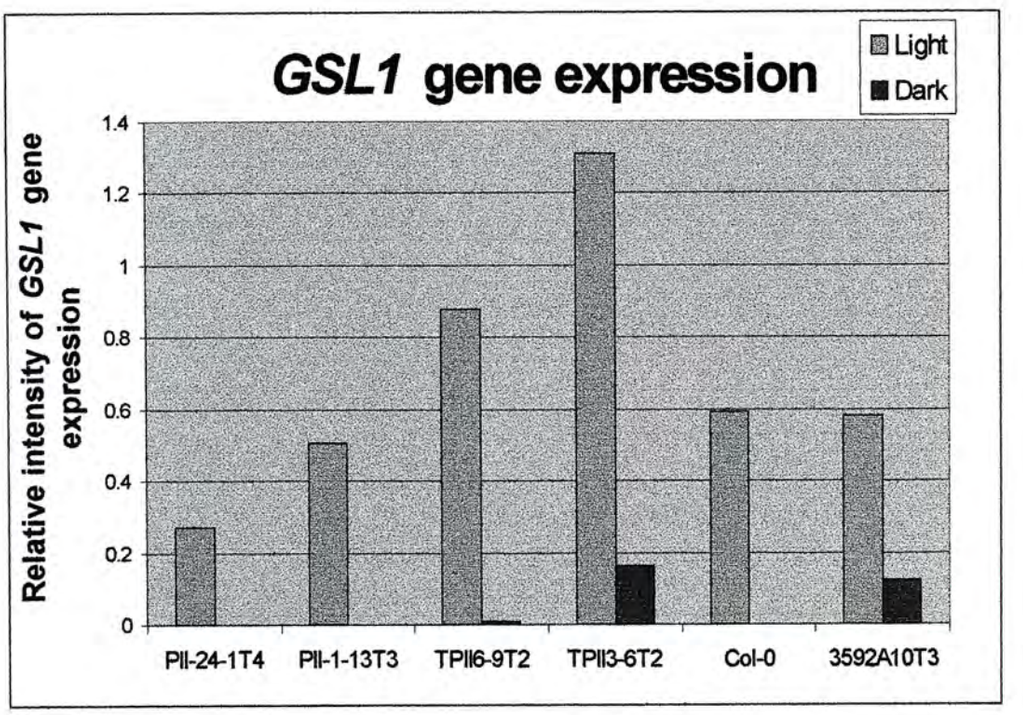

GSL1 gene expression
Light
Dark
Relative intensity of GSL1 gene expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PII-24-1T4
PII-1-13T3
TPII6-9T2
TPII3-6T2
Col-0
3592A10T3

Table 3.11: Relative quantitation of the expression of *GSL1* gene.

Constructs	Growth condition	*GSL1* signal intensity	28s RNA signal intensity	Relative intensity (X1000)	Fold changes
PII24-1T4	Light	0.26	9615	0.03	0.46
PII24-1T4	Dark	0.00	7772	0.00	0.00
PII1-13T3	Light	0.62	12301	0.05	0.85
PII1-13T3	Dark	0.00	8832	0.00	0.00
TPII6-9T2	Light	1.00	11653	0.09	1.50
TPII6-9T2	Dark	0.01	9094	0.00	0.01
TPII3-6T2	Light	1.20	9477	0.13	2.20
TPII3-6T2	Dark	0.11	6861	0.02	0.27
Col-0	Light	0.56	9452	0.06	1.00
Col-0	Dark	0.00	7416	0.00	0.00
359.2A10T3	Light	0.70	12124	0.06	0.98
359.2A10T3	Dark	0.11	9162	0.01	0.21

results of the intensity of *GSL1* hybridization signal normalized to rRNA signal on ethidium bromide gel were presented in Figure 3.13B and Table 3.11.

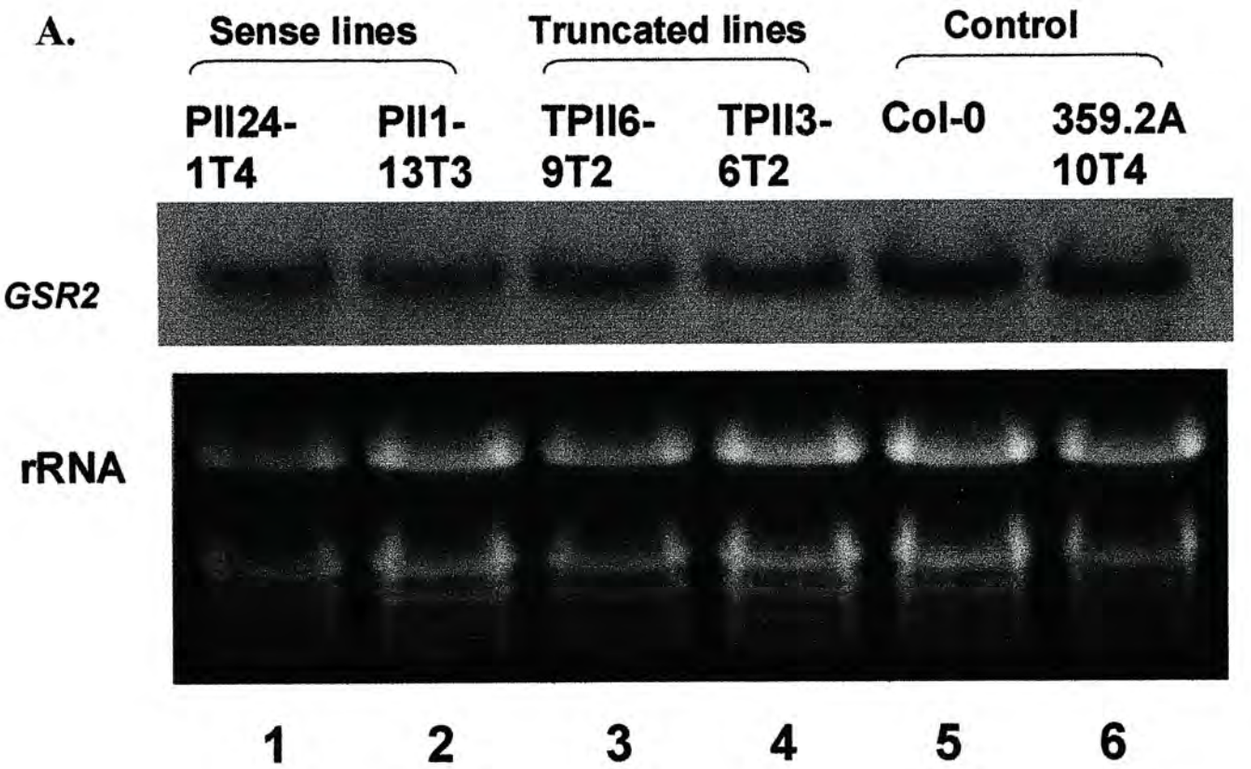
On the other hand, all lines showed similar expression levels for the cytoplasmic GS gene (*GSR2*) (Figure 3.14A). The results of the intensity of *GSR2* hybridization signal normalized to rRNA signal on ethidium bromide gel were presented in Figure 3.14B and Table 3.12.

3.4.3 Asparagine synthetase

Northern blot analysis indicated that the steady state *ASN1* mRNA level in all lines were similar (Figure 3.15, Table 3.13). By contrast, all PII and truncated PII transgenic lines showed lower *ASN2* mRNA levels. This effect was most prominent in one of the truncated lines, TPII3-6T2 when grown in soil (Figure 3.16, Table 3.14). In a separate experiment, the plants were grown under regular day-light cycle (see Materials and Methods) on regular MS agar plates and subsequently transferred to new MS agar plates with or without 3% sucrose supplements under 48 hours of continuous dark treatment. Consistent with previous findings, all lines showed repressed level of the *ASN2* gene expression, probably due to sucrose starvation. However, supplementation of 3% sucrose in the medium partially relieved the repression of *ASN2* gene expression in all lines except the truncated PII transgenic

Figure 3.14: Steady state levels of *GSR2* mRNA (light). (A) Results of Northern Blot analysis; (B) Intensity of *GSR2* hybridization signal normalized to rRNA signal on ethidium bromide gels. PII transgenic lines PII24-1T4 (lane 1) and PII1-13T3 (lane 2), truncated PII transgenic lines TPII6-9T2 (lane 3) and TPII3-6T2 (lane 4), wild type parent Col-0 (lane 5), and the control empty vector transformant 359.2A10T4 (lane 6) were grown on regular MS agar plates, imbibed in dark for 2 days, and subsequently grown under a regular day-night cycle for 14 days before subjected to continuous light treatment for 48 hours. Total leaf RNA was extracted and an aliquot of 15μg from each line was loaded onto the gel. DIG-labeled probes were used to detect *GSR2* mRNA. Numerical values of intensity of *GSR2* hybridization signal normalized to rRNA signal on ethidium bromide gel were shown on Table 3.12.

A.


Sense lines
Truncated lines
Control
PII24-1T4
PII1-13T3
TPII6-9T2
TPII3-6T2
Col-0
359.2A 10T4
GSR2
rRNA
1
2
3
4
5
6

B.

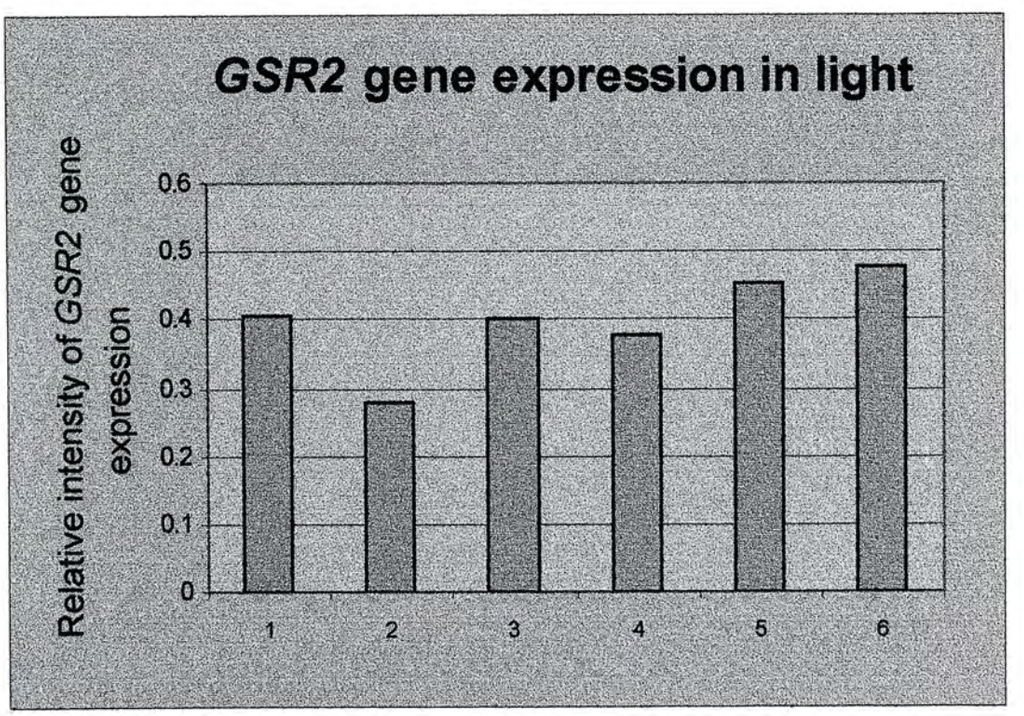
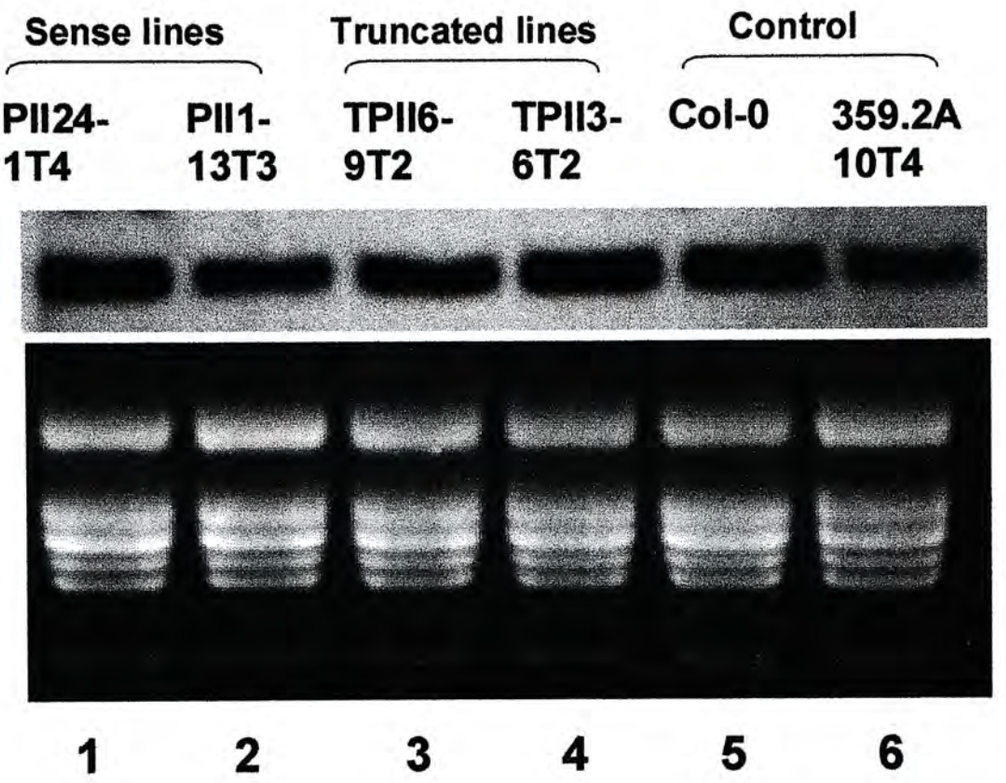

GSR2 gene expression in light
Relative intensity of GSR2 gene expression
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6

Table 3.12: Relative quantitation of the expression of *GSR2* gene (light).

Constructs	Growth condition	*GSR2* signal intensity	28s RNA signal intensity	Relative intensity (X1000)	Fold changes
PII-24-1T4	Light	4.70	11551	0.41	0.90
PII-1-13T3	Light	4.30	15409	0.28	0.62
TPII6-9T2	Light	5.42	13572	0.40	0.88
TPII3-6T2	Light	6.41	17021	0.38	0.83
Col-0	Light	8.21	18108	0.45	1.00
3592A10T3	Light	8.23	17168	0.48	1.06

Figure 3.15: Steady state levels of *ASN1* mRNA (dark). (A) Results of Northern Blot analysis; (B) Intensity of *ASN1* hybridization signal normalized to rRNA signal on ethidium bromide gels. PII transgenic lines PII24-1T4 (lane 1) and PII1-13T3 (lane 2), truncated PII transgenic lines TPII6-9T2 (lane 3) and TPII3-6T2 (lane 4), wild type parent Col-0 (lane 5), and the control empty vector transformant 359.2A10T4 (lane 6) were grown on regular MS agar plates, imbibed in dark for 2 days, and subsequently grown under a regular day-night cycle for 14 days before subjected to continuous light treatment for 48 hours. Total leaf RNA was extracted and an aliquot of 15μg from each line was loaded onto the gel. DIG-labeled probes were used to detect *ASN1* mRNA. Numerical values of intensity of *ASN1* hybridization signal normalized to rRNA signal on ethidium bromide gel were shown on Table 3.13.

A.


Sense lines
Truncated lines
Control
PII24-1T4
PII1-13T3
TPII6-9T2
TPII3-6T2
Col-0
359.2A 10T4
ASN1
rRNA
1
2
3
4
5
6

B.

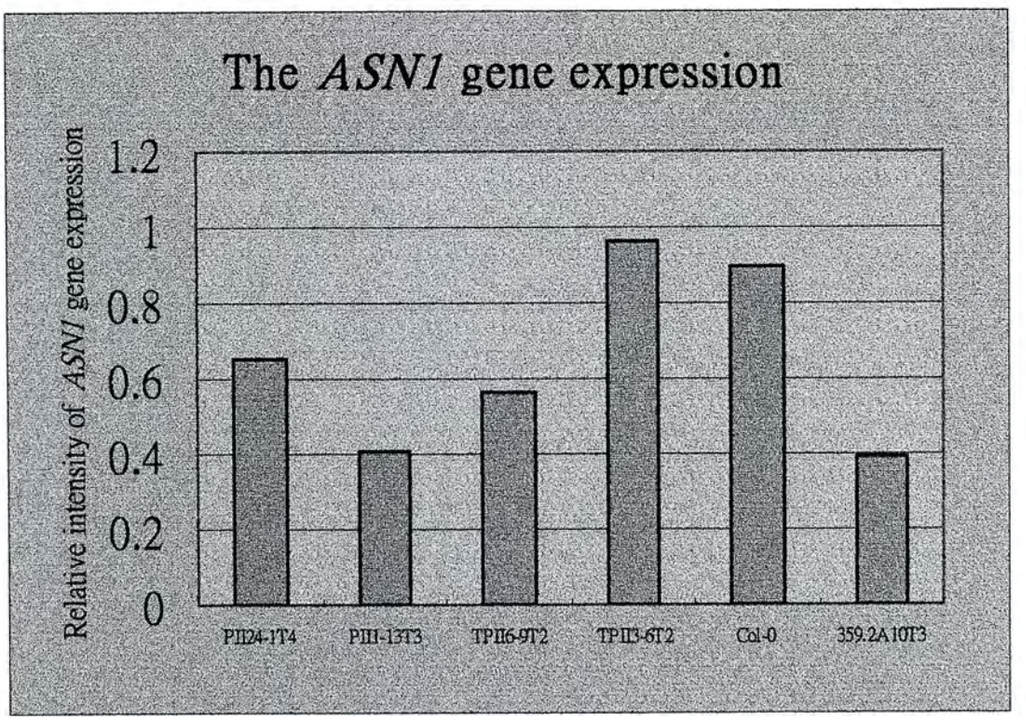
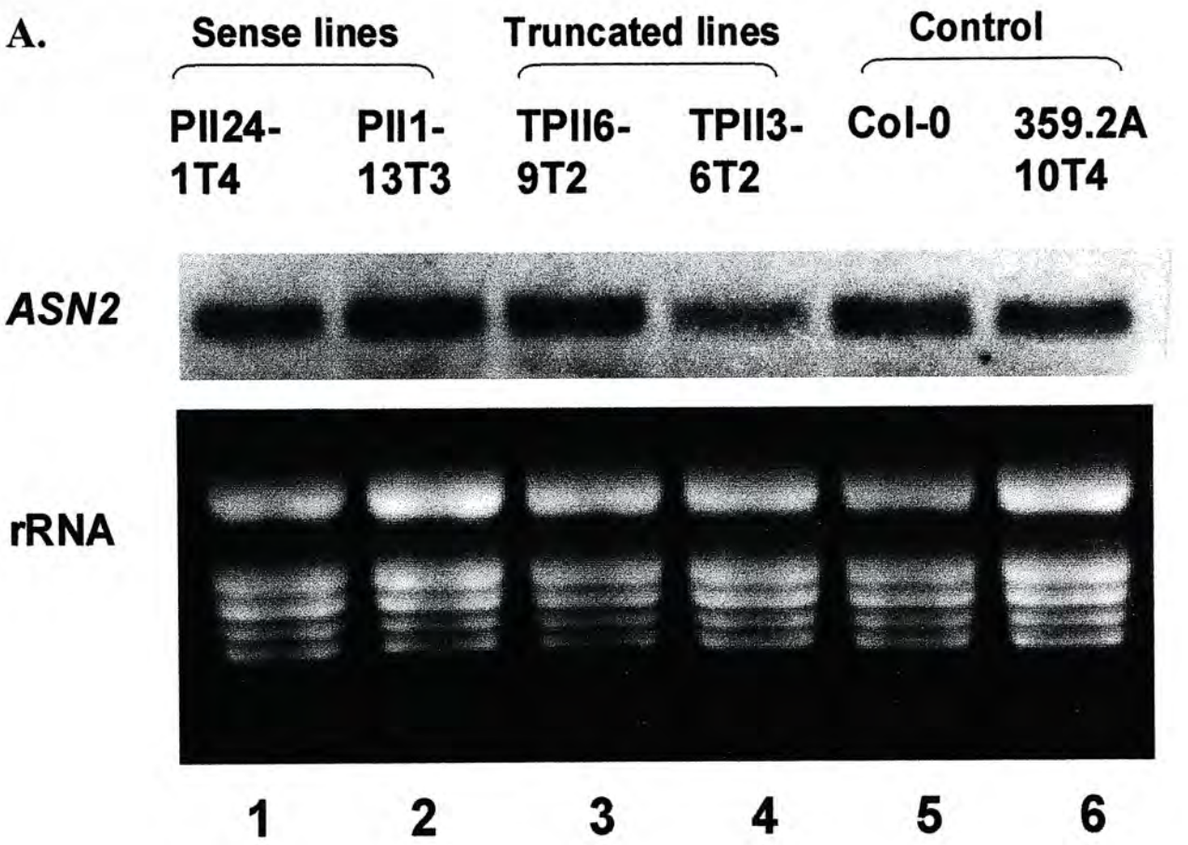

The ASN1 gene expression
Relative intensity of ASN1 gene expression
1.2
1
0.8
0.6
0.4
0.2
0
PII24-1T4
PII1-13T3
TPII6-9T2
TPII3-6T2
Col-0
359.2A10T3

Table 3.13: Relative quantitation of the expression of *ASN1* gene (dark).

Constructs	Growth condition	*ASN1* signal intensity (2 sig. fig.)	28s RNA signal intensity	Relative intensity (X1000) (2 sig. fig.)	Fold changes (2 sig. fig.)
PII-24-1T4	Dark	5.20	7950	0.65	0.73
PII-1-13T3	Dark	3.60	8805	0.41	0.46
TPII6-9T2	Dark	4.30	7574	0.56	0.63
TPII3-6T2	Dark	6.60	6884	0.96	1.10
Col-0	Dark	6.20	6965	0.90	1.00
3592A10T3	Dark	3.20	8340	0.39	0.43

Figure 3.16: Steady state levels of *ASN2* mRNA (light). (A) Results of Northern Blot analysis; (B) Intensity of *ASN2* hybridization signal normalized to rRNA signal on ethidium bromide gels. PII transgenic lines PII24-1T4 (lane 1) and PII1-13T3 (lane 2), truncated PII transgenic lines TPII6-9T2 (lane 3) and TPII3-6T2 (lane 4), wild type parent Col-0 (lane 5), and the control empty vector transformant 359.2A10T4 (lane 6) were grown on regular MS agar plates, imbibed in dark for 2 days, and subsequently grown under a regular day-night cycle for 14 days before subjected to continuous light treatment for 48 hours. Total leaf RNA was extracted and an aliquot of 15μg from each line was loaded onto the gel. DIG-labeled probe were used to detect *ASN2* mRNA. Numerical values of intensity of *ASN2* hybridization signal normalized to rRNA signal on ethidium bromide gel were shown on Table 3.14.

A.


Sense lines
Truncated lines
Control
PII24-1T4
PII1-13T3
TPII6-9T2
TPII3-6T2
Col-0
359.2A 10T4
ASN2
rRNA
1
2
3
4
5
6

B.

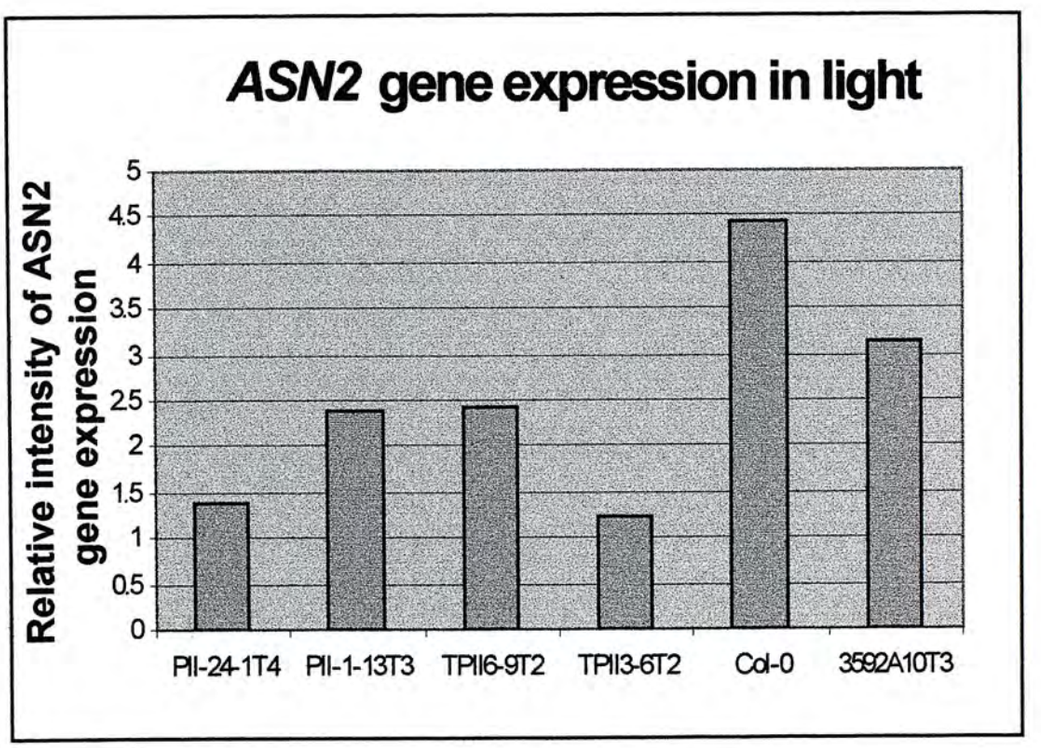

ASN2 gene expression in light
Relative intensity of ASN2 gene expression
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
PII-24-1T4
PII-1-13T3
TPII6-9T2
TPII3-6T2
Col-0
3592A10T3

Table 3.14: Relative quantitation of the expression of *ASN2* gene (Light).

Constructs	Growth condition	*ASN2* signal intensity (2 sig. fig.)	28s RNA signal intensity	Relative intensity (X1000) (2 sig. fig.)	Fold changes (2 sig. fig.)
PII-24-1T4	Light	1.20	8933	0.14	0.31
PII-1-13T3	Light	2.70	11241	0.24	0.54
TPII6-9T2	Light	2.20	9260	0.24	0.55
TPII3-6T2	Light	1.10	9036	0.12	0.28
Col-0	Light	3.20	7294	0.44	1.00
3592A10T3	Light	3.20	10388	0.31	0.71

line, TPII3-6T2. Even with 3% sucrose supplement, *ASN2* mRNA was nearly undetectable in TPII3-6T2 (Figure 3.17, Table 3.15). The regulation of *ASN3* gene expression is still pretty much unknown. However, very low levels of *ASN3* mRNA were observed in all lines harvested after 48 hours of continuous light treatment. Although barely distinguishable, it appeared that both the PII transgenic lines (PII24-1T4 & PII1-13T3) and truncated PII transgenic lines (TPII3-6T2 & TPII6-9T2) showed lower *ASN3* mRNA levels when compared to the control plants (Col-0 & 3592A10T3) (Figure 3.18, Table 3.16).

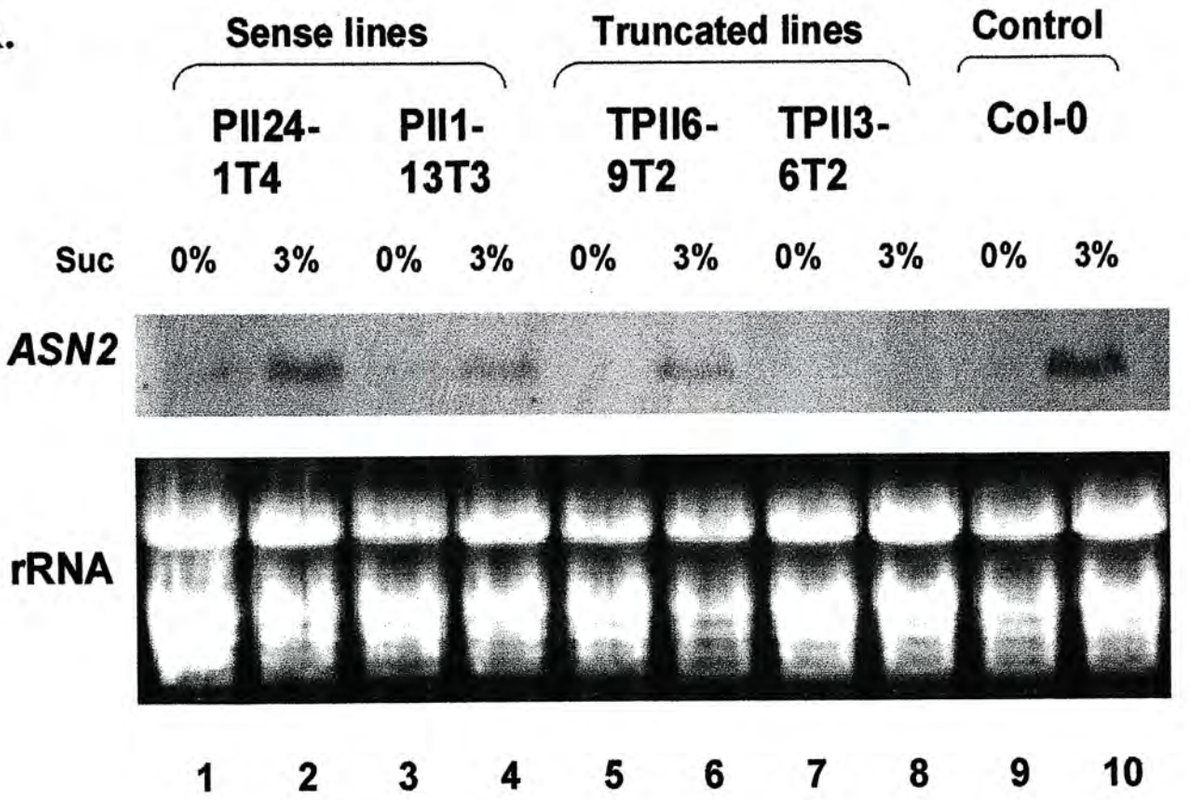
3.5 Total glutamine synthetase enzyme activity

In bacteria, PII regulates not only the transcriptional of the Gln operon (encoding GS) but also the GS enzyme activities. In *Arabidopsis thaliana*, at least four GS isozymes were found. In this research, total glutamine synthetase enzyme activities of crude extracts were measured.

Ten-day-old seedlings with similar root length originally grown on regular MS agar plates were transferred to the soil for a further growth of 15 days. At the end of the growth period, the plants were subjected to a 2-day light treatment before harvesting. Rosette leaves of the plants were cut and crude extraction containing total GS was obtained (see Materials and Methods), before measuring the specific enzyme

Figure 3.17: Steady state levels of *ASN2* mRNA (dark). (A) Results of Northern blot analysis; (B) Intensity of *ASN2* hybridisation signal normalized to rRNA signal on ethidium bromide gels. Reduction of *ASN2* mRNA levels in both PII overexpressing lines and truncated PII overexpressing lines. PII transgenic lines PII24-1T4 (lanes 1 and 2) and PII1-13T3 (lanes 3 and 4), truncated PII transgenic lines TPII6-9T2 (lanes 5 and 6) and TPII3-6T2 (lanes 7 and 8), wild type parent Col-0 (lanes 9 and 10) were grown on MS agar plates, imbibed in dark for 2 days, and subsequently grown under a regular day-light cycle for 14 days before subjected to new MS agar plates with (lanes 2, 4, 6, 8 and 10) or without (lanes 1,3, 5, 7 and 9) 3% sucrose supplement for 48 hours of continuous darkness. Total leaf RNA was extracted and an aliquot of 15μg from each line was loaded onto the gel. DIG-labeled probes were used to detect *ASN2* mRNA. Numerical values of the intensity of *ASN2* hybridization signal normalized to rRNA signal on ethidium bromide gel were shown on Table 3.15.

A.


Sense lines
Truncated lines
Control
PII24-1T4
PII1-13T3
TPII6-9T2
TPII3-6T2
Col-0
Suc 0% 3% 0% 3% 0% 3% 0% 3% 0% 3%
ASN2
rRNA
1 2 3 4 5 6 7 8 9 10

B.

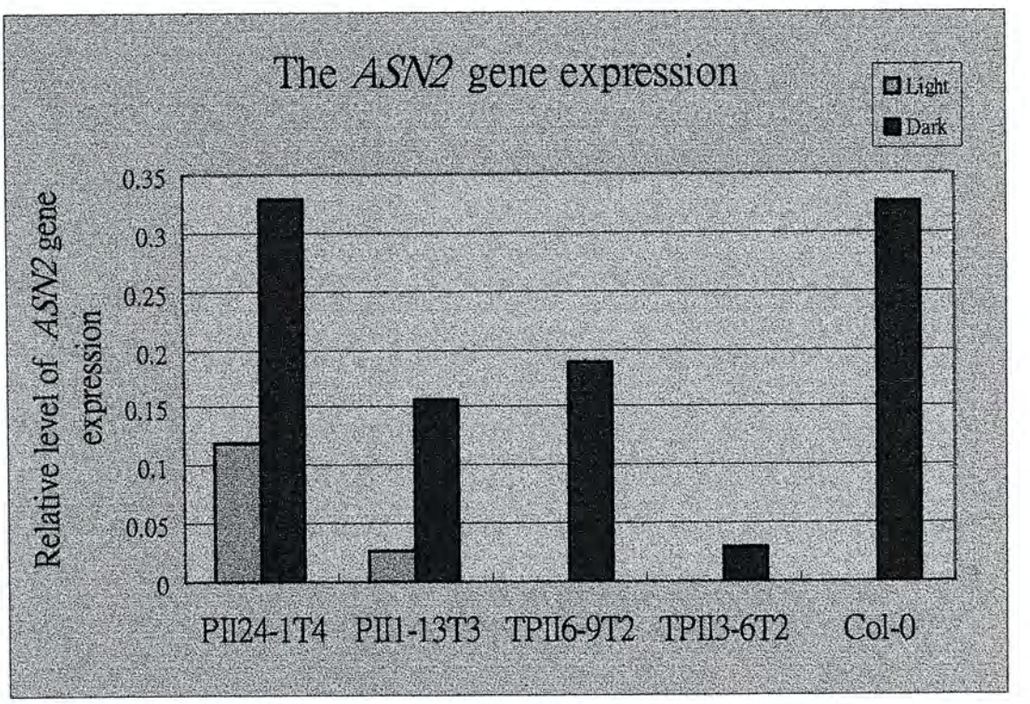
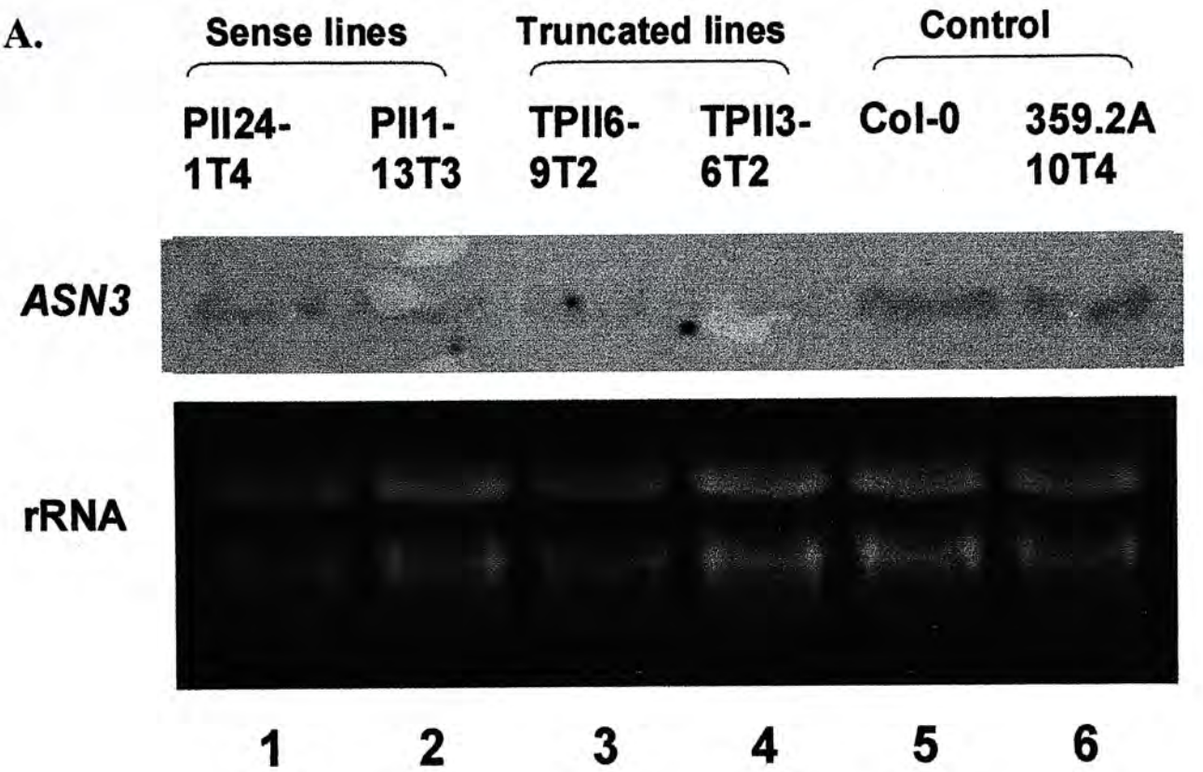

The ASN2 gene expression
Light
Dark
Relative level of ASN2 gene expression
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
PII24-1T4
PII1-13T3
TPII6-9T2
TPII3-6T2
Col-0

Table 3.15: Relative quantitation of the expression of *ASN2* gene (Dark).

Constructs	Growth condition	*ASN2* signal intensity	28s RNA signal intensity	Relative intensity (X1000)	Fold changes
PII24-1T4	Dark / 0% suc	1.72	14377	0.12	0.37
PII24-1T4	Dark / 3% suc	4.11	12501	0.33	1.01
PII1-13T3	Dark / 0% suc	0.32	12318	0.03	0.08
PII1-13T3	Dark / 3% suc	1.99	12707	0.16	0.48
TPII6-9T2	Dark / 0% suc	0.00	11208	0.00	0.00
TPII6-9T2	Dark / 3% suc	2.03	10740	0.19	0.58
TPII3-6T2	Dark / 0% suc	0.17	11784	0.00	0.00
TPII3-6T2	Dark / 3% suc	0.39	13555	0.03	0.089
Col-0	Dark / 0% suc	0.00	9648	0.00	0.00
Col-0	Dark / 3% suc	4.14	12739	0.32	1.00

Figure 3.18: Steady state levels of *ASN3* mRNA (light). (A) Results of Northern Blot analysis; (B) Intensity of *ASN3* hybridization signal normalized to rRNA signal on ethidium bromide gels. PII transgenic lines PII24-1T4 (lane 1) and PII1-13T3 (lane 2), truncated PII transgenic lines TPII6-9T2 (lane 3) and TPII3-6T2 (lane 4), wild type parent Col-0 (lane 5), and the control empty vector transformant 359.2A10T4 (lane 6) were grown on regular MS agar plates, imbibed in dark for 2 days, and subsequently grown under a regular day-night cycle for 14 days before subjected to continuous light treatment for 48 hours. Total leaf RNA was extracted and an aliquot of 15μg from each line was loaded onto the gel. DIG-labeled probe were used to detect *ASN3* mRNA. Numerical values of intensity of *ASN3* hybridization signal normalized to rRNA signal on ethidium bromide gel were shown on Table 3.16.

A.


Sense lines
Truncated lines
Control
PII24-1T4
PII1-13T3
TPII6-9T2
TPII3-6T2
Col-0
359.2A 10T4
ASN3
rRNA
1
2
3
4
5
6

B.

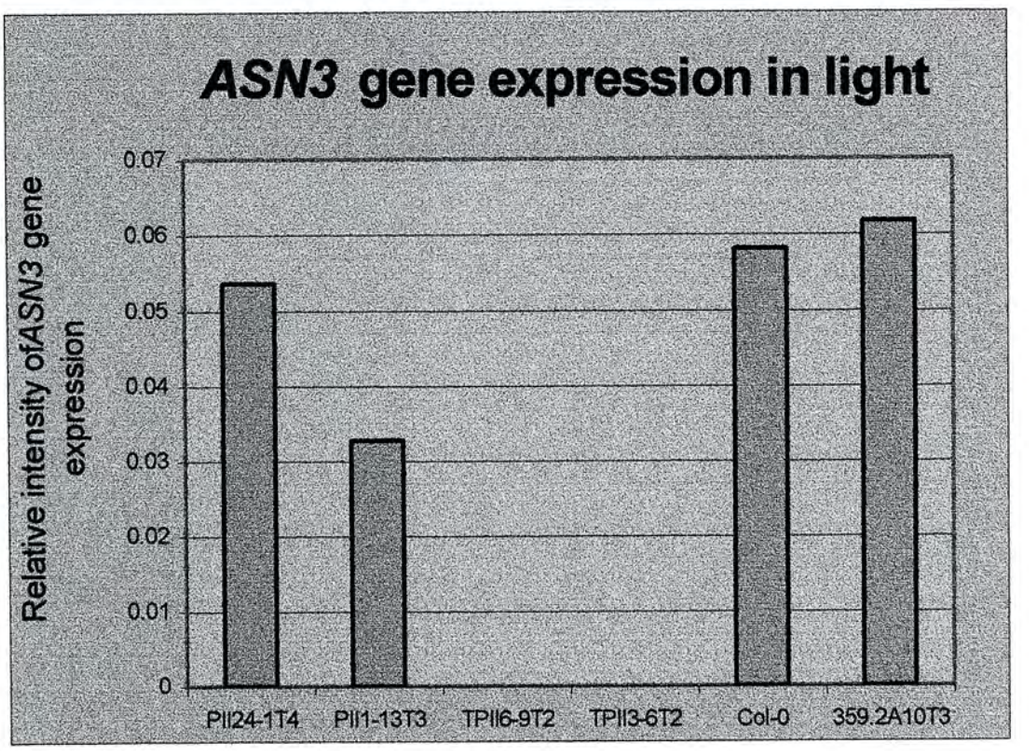

ASN3 gene expression in light
Relative intensity of ASN3 gene expression
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
PII24-1T4
PII1-13T3
TPII6-9T2
TPII3-6T2
Col-0
359.2A10T3

Table 3.16: Relative quantitation of the expression of *ASN3* gene (light).

Constructs	Growth condition	*ASN3* signal intensity (2 sig. fig.)	28s RNA signal intensity	Relative intensity (X1000) (2 sig. fig.)	Fold changes (2 sig. fig.)
PII-24-1T4	Light	0.54	10098	0.05	0.92
PII-1-13T3	Light	0.44	13497	0.03	0.56
TPII6-9T2	Light	0.00	10222	0.00	0.00
TPII3-6T2	Light	0.00	14175	0.00	0.00
Col-0	Light	0.80	14229	0.06	1.00
3592A10T3	Light	0.86	13876	0.06	1.10

activities (see Materials and Methods). While PII24-1T4 and TPII3-6T2 showed slightly lower total GS activities (Figure 3.19, lanes 1 and 4), other lines exhibited similar total activities as compared to Col-0 (Figure 3.19, lanes 2, 3, 5 & 6).

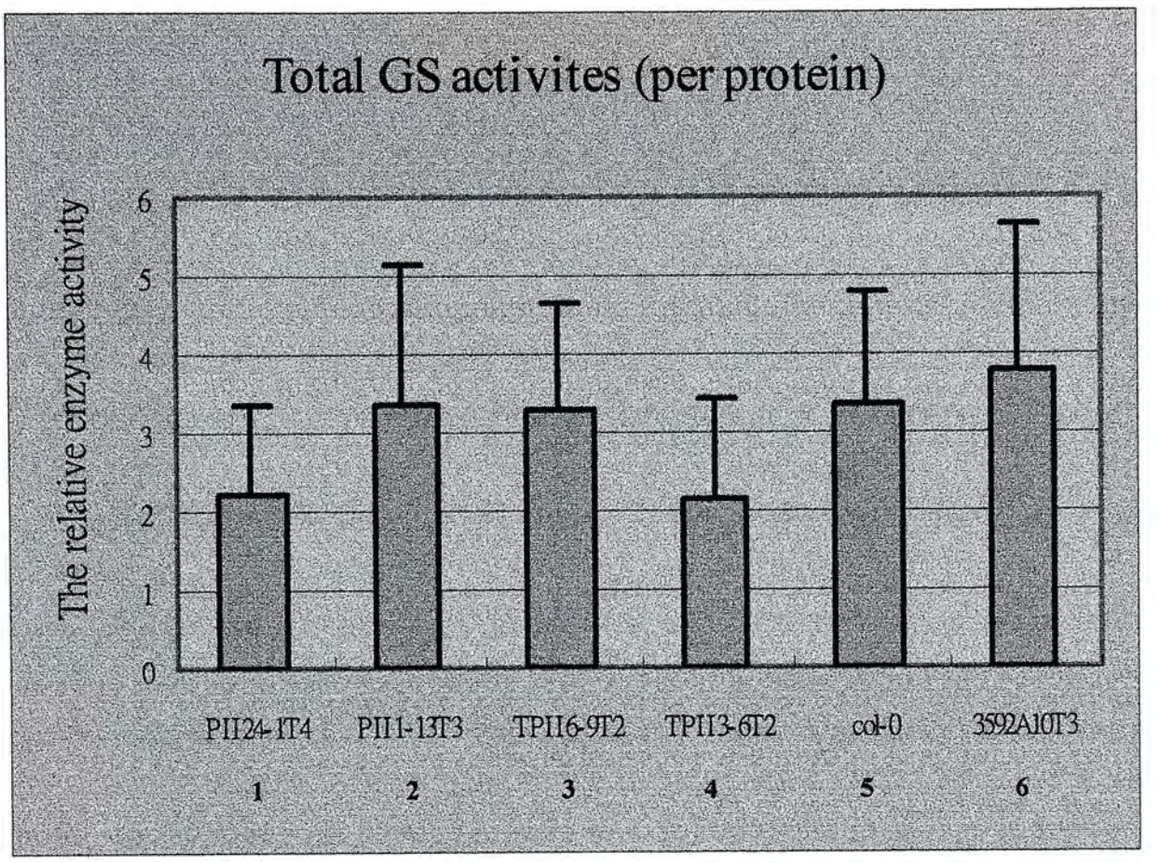


Figure 3.19: Total GS activities (per protein). Seeds of PII transgenic lines (lanes 1 and 2), truncated PII transgenic lines (lanes 3 and 4), wild type Col-0 (lane 5) and empty vector transformant control 359.2A10T3 (lane 6) were sown on regular MS agar plates supplemented with 3% sucrose, imbibed in dark for 2 days, and allowed to further grow for 10 days. Seedlings of similar root length were transferred to soil and grown for 15 days before harvesting. Enzyme extraction and activity assay were performed as described in Materials and Methods.

4. Discussion

PII protein plays a central role in nitrogen sensing in bacteria and probably is one of the most highly conserved signaling molecules (Merrick and Edwards, 1995, Hsieh *et al.*, 1998). Recently, it was found that PII not only exist in bacteria but also in various higher organisms such as red algae, alfalfa, caster bean and *Arabidopsis thaliana*. Before the discovery of plant PIIs, no common nitrogen regulating mechanism was found among bacteria, algae and higher plants, nor any nitrogen regulating mechanisms and signaling pathways was reported in higher plants. The newly discovered PII-like protein found in *Arabidopsis thaliana* shed light to the understanding of the yet unknown nitrogen sensing pathway in higher plants. In this study, previously constructed PII overexpressing and truncated PII overexpressing transgenic lines were characterized, in comparison to the wild type parent Col-0 and empty vecter transformant control. The experimental results were summarized in Table 4.1.

4.1 Overexpressing PII and truncated PII in the transgenic plants

Northern blot analysis indicated that the transgenic plants successfully overexpress *PII (GLB1)* and truncated *PII (GLB1)*. It is interesting to note that even though both ,

Table 4.1: Summary of the experiment results

	Experiments	Constructs		Results	References
Construction of transgenic plants	Constructions and verification of the PII and truncated PII transgenic plants	PII overexpressing transgenic lines	PII24-1T4	Sucessfully overproducing both intact *PII (GLB1)* mRNA and PII protein.	Heish *et al.*, 1998
			PII1-13T3		
		Truncated PII overexpressing transgenic lines	TPII6-9T2	Sucessfully overproducing both truncated *PII (GLB1)* mRNA and PII protein.	Heish *et al.*, unpubished data
			TPII3-6T2		
		Controls	Col-0	Wild type parent	
			359.2A10T3	Vector alone transformant	
Physiology	General growth characteristics when grown on soil without any fertilizers added	PII overexpressing transgenic lines	PII24-1T4	Both lines seemed to be of similar size and thicker stems with controls.	This work, Figure 3.3
			PII1-13T3		
		Truncated PII overexpressing transgenic lines	TPII6-9T2	It bolted much earlier than controls with thinner stems and small-size leaves.	
			TPII3-6T2	It grew much slower than controls with delayed flowering time.	
		Controls	Col-0	They grew healthy with abundant flowers and siliques.	
			359.2A10T3		

Physiology	Chlorophyll contents of shoots when grown on soil (per leaf disc)	PII overexpressing transgenic lines	PII24-1T4	No significant difference with Col-0.	This work, Figure 3.6
			PII1-13T3		
		Truncated PII overexpressing transgenic lines	TPII6-9T2		
			TPII3-6T2		
		Controls	Col-0		
			359.2A10T3		
	Chlorophyll contents of shoots when grown on 3% sucrose MS agar plates with 20mM ammonium sources Supplemented	PII overexpressing transgenic lines	PII24-1T4	No significant difference with Col-0.	This work, Figure 3.5
			PII1-13T3		
		Truncated PII overexpressing transgenic lines	TPII6-9T2		
			TPII3-6T2	Significantly lower chlorophyll contents than Col-0.	
		Controls	Col-0	Control	
			359.2A10T3	No significant difference with Col-0.	
	Chlorophyll contents of shoots when grown on 3% sucrose MS agar plates without ammonium sources supplemented	PII overexpressing transgenic lines	PII24-1T4	Significantly lower chlorophyll contents than Col-0.	This work, Figure 3.7
			PII1-13T3		
		Truncated PII overexpressing transgenic lines	TPII6-9T2	No significant difference with Col-0.	
			TPII3-6T2	Significantly lower chlorophyll contents than Col-0.	
		Controls	Col-0	Control	
			359.2A10T3	Significantly lower chlorophyll contents than Col-0.	

Physiology	Chlorophyll contents of shoots when grown on 3% sucrose MS agar plates with 100mM ammonium sources supplemented	PII overexpressing transgenic lines	PII24-1T4	Both lines have significantly higher chlorophyll contents than Col-0.	This work, Figure 3.7
			PII1-13T3		
		Truncated PII overexpressing transgenic lines	TPII6-9T2	No significant difference with Col-0.	
			TPII3-6T2		
		Controls	Col-0		
			359.2A10T3		
	Root growth on regular MS agar plates	PII overexpressing transgenic lines	PII24-1T4	No significant difference with Col-0.	This work, Figure 3.8
			PII1-13T3		
		Truncated PII overexpressing transgenic lines	TPII6-9T2		
			TPII3-6T2		
		Controls	Col-0		
			359.2A10T3	N.D.	
	Root growth on nitrogen-free agar plates	PII overexpressing transgenic lines	PII24-1T4	No significant difference with Col-0.	This work, Figure 3.9
			PII1-13T3		
		Truncated PII overexpressing transgenic lines	TPII6-9T2	Significantly longer root length with Col-0.	
			TPII3-6T2		
		Controls	Col-0	No significant difference with Col-0.	
			359.2A10T3		
	Nitrogen contents in the seeds	PII overexpressing transgenic lines	PII24-1T4	Similar to Col-0.	This work, Figure 3.10
			PII1-13T3		
		Truncated PII overexpressing transgenic lines	TPII6-9T2	Slightly higher percentage of nitrogen and nitrogen to carbon ratio in seeds.	
			TPII3-6T2		
		Controls	Col-0	Control	
			359.2A10T3	Similar to Col-0.	

Nitrogen assimilatory gene expressions	*NIA1* (encoding for nitrate reductase) steady state expression level	PII overexpressing transgenic lines	PII24-1T4	Reduced expression levels than Col-0, especially in the truncated lines.	This work, Figure 3.11 and Table 3.9
			PII1-13T3		
		Truncated PII overexpressing transgenic lines	TPII6-9T2		
			TPII3-6T2		
		Controls	Col-0	Control	
			359.2A10T3	Similar expression level with Col-0.	
	NIA2 (encoding for nitrate reductase) steady state expression level	PII overexpressing transgenic lines	PII24-1T4	Reduced expression levels than Col-0, especially in case of TPII3-6T2 and PII24-1T4.	This work, Figure 3.12 and Table 3.10
			PII1-13T3		
		Truncated PII overexpressing transgenic lines	TPII6-9T2		
			TPII3-6T2		
		Controls	Col-0	Control	
			359.2A10T3	Similar expression level with Col-0.	
	GSL1 (encoding for the only chloroplastic GS) steady state expression level	PII overexpressing transgenic lines	PII24-1T4	Similar expression levels with Col-0.	This work, Figure 3.13 and Table 3.11
			PII1-13T3		
		Truncated PII overexpressing transgenic lines	TPII6-9T2	Slightly higher gene expression than Col-0.	
			TPII3-6T2		
		Controls	Col-0	Control	
			359.2A10T3	Similar expression level with Col-0.	
	GSR2 (encoding for one of the cytoplastic GS) steady state expression level (Light)	PII overexpressing transgenic lines	PII24-1T4	Similar expression levels with Col-0.	This work, Figure 3.14 and Table 3.12
			PII1-13T3		
		Truncated PII overexpressing transgenic lines	TPII6-9T2		
			TPII3-6T2		
		Controls	Col-0		
			359.2A10T3		

Nitrogen assimilatory gene expressions	*ASN1* (encoding for asparagine synthetase) steady state expression level (Dark)	PII overexpressing transgenic lines	PII24-1T4	Similar gene expression levels with Col-0.	This work, Figure 3.15 and Table 3.13
			PII1-13T3		
		Truncated PII overexpressing transgenic lines	TPII6-9T2		
			TPII3-6T2		
		Controls	Col-0		
			359.2A10T3		
	ASN2 (encoding for asparagine synthetase) steady state expression level (Light)	PII overexpressing transgenic lines	PII24-1T4	Reduced expression levels than Col-0, especially in TPII3-6T2.	This work, Figure 3.16 and Table 3.14
			PII1-13T3		
		Truncated PII overexpressing transgenic lines	TPII6-9T2		
			TPII3-6T2		
		Controls	Col-0	Control	
			359.2A10T3	Similar expression level with Col-0.	
	ASN2 steady state expression level (Dark)	PII overexpressing transgenic lines	PII24-1T4	Sucrose induction of *ASN2* expression in dark but with reduced levels than Col-0.	This work, Figure 3.17 and Table 3.15
			PII1-13T3		
		Truncated PII overexpressing transgenic lines	TPII6-9T2		
			TPII3-6T2	Sucrose induction on the *ASN2* expression was nearly abolished in dark.	
		Controls	Col-0	Sucrose induced the *ASN2* expression in dark.	
			359.2A10T3	N.D.	
	ASN3 (encoding for asparagine synthetase) steady state expression level (Light)	PII overexpressing transgenic lines	PII24-1T4	Slightly reduced expression levels than Col-0.	This work, Figure 3.18 and Table 3.16
			PII1-13T3		
		Truncated PII overexpressing transgenic lines	TPII6-9T2	No detectable expression levels.	
			TPII3-6T2		
		Controls	Col-0	Control	
			359.2A10T3	Similar expression level with Col-0.	

Enzyme activities				
Total glutamine synthetase enzyme activity	PII overexpressing transgenic lines	PII24-1T4	Slightly lowered GS enzyme activity.	This work, Figure 3.19
		PII1-13T3	Similar activity levels with Col-0.	
	Truncated PII overexpressing transgenic lines	TPII6-9T2		
		TPII3-6T2	Slightly lowered GS enzyme activity.	
	Controls	Col-0	Control	
		359.2A10T3	Similar activity level with Col-0.	

the truncated lines overexpressed the truncated *PII (GLB1)* mRNA when grown in soil, TPII3-6T2 showed a higher expression level than TPII6-9T2. Such difference in the expression level may explain some differences between the two truncated PII transgenic lines in respect to gene expressions and general growth in soil. Surprisingly, when growth in full strength MS agar plates, the expression levels of the truncated *PII(GLB1)* mRNA in both TPII6-9T2 and TPII3-6T2 were similar. Possible reasons for such difference may be that MS agar plates contained more carbon resources (sucrose) than the soil or that the different developmental stage of the tested plants in MS agar plates (10-day-old) and in soil (24-day-old) that affect the truncated *PII (GLB1)* mRNA expression in the transgenic plants.

Northern blot analyses also showed that when grown in soil, both the PII overexpressing transgenic lines overexpress the *PII* (*GLB1*) mRNA together with two mRNA species of smaller size. Both the truncated PII overexpressing lines overexpress the truncated *PII (GLB1)* mRNA together with the native *PII (GLB1)* mRNA. When grown in soil, TPII6-9T2 showed decreased level of native *PII (GLB1)* mRNA and high levels of truncated *PII (GLB1)* mRNA. However, TPII3-6T2 did not showed any decrease in the level of native *PII (GLB1)* mRNA. Instead, it showed elevated level of both native mRNA and truncated *PII (GLB1)* mRNA.

4.2 The overall growth and development

Retarded growth was observed in both truncated PII transgenic lines. TPII6-9T2 exhibited small leaves, thin stems and early flowering phenotype. On the other hand, TPII3-6T2 showed delayed growth and development although no small leaves and thin stems were observed. Both the truncated lines overproduce the truncated PII proteins that may incorporated with intact PII protein to form the non-functional PII proteins that ultimately lead to the disruption of normal PII function. The above observations suggest that disruption of normal PII function may have a negative consequence to plant growth and development.

4.3 Chlorophyll

When grown on 3% sucrose regular MS agar plates, TPII3-6T2 suffered a severe chlorosis and exhibited significantly lower chlorophyll contents than other lines. Plants required nitrogen for synthesis of amino acids, proteins, chlorophyll and nucleic acids and nitrogen deficiency in plants has been shown to cause a decrease in the levels of photosynthetic components such as chlorophyll and ribulose bisphosphate carboxylase, leading to reduction in photosynthetic capacity and carboxylation efficency (Delgado *et al.*, 1998). The resulting chlorosis in TPII3-6T2

may reflect the deregulation of nitrogen assimilation in at least one truncated line and such deregulation is so severe that it affects the synthesis of chlorophyll. However, further experiments are required to prove it.

On the other hand, nitrogen in form of ammonia is toxic to plants. In this study, PII transgenic plants were shown to have a higher tolerance against 100mM ammonium nitrate. When the plants were grown with sufficient nitrate but no ammonium supply, the PII overexpressing transgenic lines (PII24-1T4 and PII1-13T3) showed significantly lower chlorophyll concentration than that of Col-0. When ammonium concentration increased to 20mM (i.e. as in full strength MS plates), no significant difference was found between the chlorophyll content of the PII overexpressing transgenic lines and Col-0.

The chlorophyll contents of all plants grown in 20mM ammonium supplement were higher than that with no ammonium supplement. When the ammonium concentration increased to 100mM, all plants showed decreased growth in root length and reduced chlorophyll synthesis. However, under such high ammonium stress, the PII overexpressing transgenic lines, PII24-1T4 and PII1-13T3, showed a less severe inhibition on the chlorophyll synthesis. They were more tolerant to the ammonium stress. One possible reason may be that PII overexpressing transgenic lines have altered ammonium sensing or even in the ability in uptaking the nitrogen sources.

When grown in ammonium-free MS agar medium, PII overexpressing transgenic lines may be less capable of uptaking or sensing nitrogen, leading to the decline in chlorophyll synthesis.

When the ammonium concentration reaches 20mM, nitrogen is not a limiting factor for growth of *Arabidopsis thaliana*. PII overexpressing transgenic lines then grew as healthy as Col-0. No significant difference in the chlorophyll concentration was seen. In 100mM ammonium nitrate, PII overexpressing transgenic lines showed the highest tolerance to ammonium stress.

4.4 Root length

Difference in the root length was observed only under nitrogen starvation but not in growth medium with sufficient nitrogen supply. When nitrogen was limiting, the truncated lines showed significantly longer root length than that of Col-0 and other lines.

Such phenomenon may be explained by the differential nitrogen storage in the seeds of the PII and truncated overexpressing transgenic plants. As the growth medium did not contain any nitrogen sources, the seeds germinate and grow solely dependent on the food storage inside the seeds. Both the percentage of nitrogen and N/C ratio of seeds of PII truncated lines were higher than other lines. As a result, truncated lines

may have higher nitrogen reserve for the root growth and hence are less sensitive to root growth inhibition under nitrogen starvation.

4.5 Expression of nitrogen assimilatory genes

Previous researches found that most genes involved in nitrogen assimilation are carefully regulated by external and internal factors such as light, metabolites and cell types. These genes may employ yet unknown common or related signaling pathways sensing the internal nitrogen and/or carbon status in the cells. In fact, some reports claimed that the ratio of cellular carbon to nitrogen is a major player in the metabolic control of nitrogen assimilation (Lam *et al.*, 1996). To test the possible effects of PII on the expression of nitrogen assimilatory genes, the steady-state mRNA levels of *ASN1*, *ASN2*, *ASN3*, *GSL1*, *GSR2*, *NIA1* and *NIA2* in PII and truncated PII transgenic lines were examined.

4.5.1 Genes encoding nitrate reductase

Inorganic nitrogen in form of nitrate in soil is taken up by plants and reduced to ammonia via the combined activities of nitrate reductase and nitrite reductase. Nitrate reductase converts nitrate into nitrite and nitrite reductase converts the toxic nitrite into ammonium. In *Arabidopsis thaliana*, two nitrate reductase genes were

identified. (Cheng *et al.*, 1988; Wilkinson & Crawford, 1991; Wilkinson & Crawford, 1993)

The *NIA2* gene encodes 90% of total nitrate reductase activities in shoots (Wilkinson & Crawford, 1991) whereas the *NIA1* gene accounts for the remaining 10-15% of the total nitrate reductase activity in *Arabidopsis thaliana*. Both of them are expressed in shoots and roots of *Arabidopsis thaliana*. The expression of genes encoding nitrate reductase is very tightly regulated by environmental and metabolic signals (Crawford, 1995; Crawford & Arst Jr., 1993; Hoff *et al.*, 1994) with the presence of nitrate being the key regulatory factor (Cheng *et al.*, 1986).

The studies in cyanobacteria suggested that PII plays a role in the control of nitrate uptake (Hisbergues *et al.*, 1999; Lee *et al.*, 2000; Lee *et al.*, 1998) and hence it may alter the internal nitrate concentration of the cells. Since nitrate acts as an important factor for controlling expression of gene encoding nitrate reductase in plants, the PII protein in *Arabidopsis thaliana* may therefore indirectly alter the expression level of genes encoding nitrate reductase.

In this study, all PII overexpressing and truncated PII overexpressing transgenic lines showed a prominent reduction in the expression of *NIA1* and *NIA2* genes, especially in one of the truncated lines, TPII3-6T2. Such effects on *NIA1* and *NIA2* gene expression may be due to the altered ability of the transgenic plants in nitrate uptake.

However, such hypothesis needs to be proven.

Besides nitrate, other factors, including light and carbohydrate, also enhance the expression of nitrate reductase, whereas reduced forms of nitrogen, in particular glutamine, down-regulate its expression in plants (Cheng *et al.*, 1992; Vincentz *et al.*, 1993). Further experiments are required to carry out to test which of the above mentioned factors and combinations of factors is responsible for the prominent reduction of *NIA1* and *NIA2* steady-state mRNA level in the transgenic plants.

4.5.2 Genes encoding glutamine synthetase

The enzyme glutamine synthetase catalyzes the ATP-dependent conversion of glutamate to glutamine using ammonia as a substrate. The four GS genes in *Arabidopsis thaliana* are differentially expressed in leaves, roots and germinating seeds. Expression of the chloroplastic GS (*GSL1*) is leave-specifc and light-regulated (Peterman & Goodman, 1991). It may function in primary ammonia assimilation as well as reassimilation of photorespiratory ammonia (Wallsgrove *et al.*, 1983, Kozaki and Takeba, 1996, Eckes *et al.*, 1989, Wallsgrove *et al.*, 1987).

The remaining three genes encoding the cytosolic form of GS. One of them, *GSR2*, exhibited the highest levels of expression in roots and much lower levels of expression in seeds and leaves (Peterman & Goodman, 1991). *GSR2* mRNA also

increases in the senescent rosette (Bernhard & Matile, 1994) and may involve in the reassimilation of ammonia resulting from the catabolism of proteins and nucleic acids (Bernhard & Matile, 1994).

Both *GSL1* and *GSR2* mRNA expression levels in the PII transgenic plants were measured. Both truncated lines, TPII6-9T2 and TPII3-6T2 showed slightly elevated level of *GSL1* when compared to Col-0. On the other hand, the *GSR2* expression is similar in all lines. Another northern blot analysis was performed and PII (*GLB1*) mRNA level is found to be similar in rosette leaves and senescent leaves. The above results may suggest that PII does not play an important role in senescence through its effect on *GSR2*. Instead, it may exert its effects on the primary nitrogen assimilation through the action of *GSL1*.

This result are consistent with the previous finding that *PII (GLB1)* mRNA level increased with chloroplastic GS but not with the cytosolic GS in Arabidopsis thaliana (Hsieh *et al.*, 1998).

4.5.3 Genes encoding asparagine synthetase

There are three *ASN* genes discovered in *Arabidopsis thaliana* (Lam *et al.*, 1996; Lam *et al.*, 1998; Lam *et al.*, 1994). The enzyme asparagine synthetase is responsible for the conversion of aspartate and glutamine into asparagine and glutamate. Such

conversion is crucial for the plants especially under the condition of carbon starvation or nitrogen excess. It is because asparagine is an ideal nitrogen carrier due to its relative stability and low carbon to nitrogen ratio. *ASN1* gene is light and sucrose repressed but it is induced in dark or by nitrogen metabolites (Lam *et al.*, 1994). Since *ASN1* gene expression is carefully regulated by carbon/nitrogen metabolites, it is possible that the ratio of organic nitrogen to carbon in a plant may be the ultimate factor controlling *ASN1* gene expression (Lam *et al.*, 1996; Lam *et al.*, 1994).

Contrasting to *ASN1*, the *ASN2* gene exhibits a reciprocal gene expression regulation in response to light and carbon/nitrogen metabolites (Lam *et al.*, 1998). Since *ASN1* and *ASN2* are expressed in different time and under different conditions, they may play unoverlapped physiological roles. Since *ASN2* is induced by several environmental stresses such as heavy metals and photorespiratory conditions (Prof.H.M.Lam, personal communication), it may function to provide the required nitrogen resources for other physiological processes (Lam *et al.*, 1996).

In parallel, it was also found that asparagine accumulates under some stress conditions, and this supports the hypothesis that *ASN2* may also play a role in the detoxification of high levels of ammonia (Sieciechowicz *et al.*, 1988). The third *ASN* gene (*ASN3*) was also found in *Arabidopsis thaliana*, *ASN3*. Its regulation and

functions remain to be clearly known.

The steady state mRNA level of all three *ASN* genes were tested. In this study, *ASN2* gene expressions were reduced in all PII overexpressing and truncated PII overexpressing lines. It was especially low in one of the truncated line, TPII3-6T2. Previous study found that 3% sucrose supplement relieves dark repression of *ASN2* gene expression in the dark-adapted wild type plants (Lam *et al.*, 1998). In this study, all lines except TPII3-6T2 exhibited such recovery of *ASN2* gene expression. In fact, no detectable signals of *ASN2* mRNA were observed in TPII3-6T2 grown on dark. These results showed clearly that severe impairment on the *ASN2* gene expression was occurred in at least one of the truncated lines. Such impairment cannot be recovered even after the addition of carbon metabolites that normally induce *ASN2* gene expression in dark.

Moreover, all PII overexpressing and truncated PII overexpressing lines as well as the control plant (Col-0 and 359.2A10T3) show low level of *ASN3* gene expression in the rosette leaves. Interestingly, all PII overexpressors and truncated PII overexpressors seemed to have lower *ASN3* mRNA levels than the controls. Since neither the exact functions nor the regulation pattern of *ASN3* are known, it is hard to delineate the relationship between PII and *ASN3* at the present time.

Together, the results of Northern blot analysis showed that PII affect a wide range of

nitrogen assimilatory genes including *NIA1*, *NIA2*, *GSL1*, *ASN2* and *ASN3*. It seemed that the overall regulation of the nitrogen assimilation in the PII transgenic plants, especially in the truncated lines, was altered.

4.6 Overall GS enzyme levels in the rosette leaves

The overall GS enzyme activities per protein and that per fresh weight were measured. The former was considered to be a more accurate and presentable results because the water content may affect the fresh weight.

PII24-1T4 and TPII3-6T2 exhibited slightly lower overall GS enzyme activities in the aerial parts than Col-0 while other lines showed similar level of GS enzyme when compared to Col-0. However, the result of enzyme activity assay is not consistent with the results of Northern blot analysis. The truncated lines, TPII6-9T2 and TPII3-6T2 had elevated level of *GSL1* and similar level of *GSR2* expression, compared to Col-0. However, the overall GS enzyme activities of both lines were not higher than that of Col-0. TPII6-9T2 showed similar level of GS enzyme activities while another truncated line, TPII3-6T2 showed a lower enzyme level.

Such inconsistency may be explained by several possibilities includes: 1) Experimental errors in enzyme activity measurement because only crude extracts were used; 2) Existence of four isoenzyme of GS genes including chloroplastic GS

gene (*GSL1*) and three cytosolic GS genes *GSR1*, *GSR2* and *GSKB*. All of them may affect the overall GS enzyme activity. However, previous studies showed that another two GS genes, *GSR1* and *GSKB* were not expressed in leaves (Peterman & Goodman, 1991). Therefore, it is not expected to exert a significant effect on the overall GS activities in leaves. Furthermore, PII is a nuclear encoded chloroplastic protein (Hsieh *et al.*, 1998). It is not expected that PII play a role in regulating *GSR1* and *GSKB* which are predominantly expressed in roots (Peterman & Goodman, 1991).

3) There may be higher number of GS subunits in the truncated lines due to the higher *GSl1* expression. However, due to unknown reasons or effects of PII, the unstable GS holoprotein was not able to form a GS enzyme complex and hence led to no increase in GS activity. In the case of TII3-6T2, such effect may be so severe that decrease in GS activity was observed. Similar phenomenon was observed in the study of soybean GS (Ireland & Lea, 1999; Temple *et al.*, 1996); 4) Post-transcriptional regulation may be involved in the regulation of GS in plants similar to that in bacteria, which results in the lowering of GS enzyme activity in the truncated lines, especially TPII3-6T2. Even though post-transcriptional regulation mechanism was yet to be discovered in plants, several studies proposed the existence of post-transcriptional and post-translational regulation of GS. A study carried out to analyze in detail the cytosolic GS mRNA and polypeptide content of rice leaves during

natural senescence (Kamachi *et al.*, 1991) revealed that chloroplastic GS mRNA increases during senescence but chloroplastic GS protein exhibits an unexpected decrease. The author proposed that post-transcriptional regulation of GS must be involved during senescence. Furthermore, another research suggested the post-translational regulation of cytosolic glutamine synthetase by reversible phosphorylation and hence regulate GS at the protein turnover level (Finnemann & Schjoerring, 2000).

4.7 N/C ratio of the seed storage

Current experimental results suggest that both the truncated lines showed slightly higher N/C ratio and higher percentage of nitrogen in seeds. It is believed that higher nitrogen resource may lead to a better growth. However, the observation of retarded growth of the truncated lines and its higher N/C ratio in seeds seemed to be contradictory. Even though the truncated lines might have higher nitrogen storage, it did not show healthy growth in the soil or full strength MS agar plates. Such results may imply that there are altered allocations of the nitrogen metabolites in the truncated PII overexpressing transgenic lines.

4.8 Proposed model for the roles of PII

To put the observation of this study into perspective, a model is proposed to explain the possible roles of PII (Figure 4.1). When nitrogen is in excess, PII exists in a modified form which then exerts positive effects on the expression of several nitrogen assimilatory genes (including *ASN2*, *ASN3*, *NIA1* and *NIA2*) and negative effect on *GSL1*. Such transcriptional regulation by PII enables the control and regulation of the global nitrogen assimilation which will directly and indirectly provide free amino acids, proteins, chlorophylls and nitrogen reserves. As a consequence, it affects the growth and development of plants.

Similarly, when the plants face nitrogen starvation, unmodified form of PII will be predominant. The unmodified form of PII exerts opposite effects on the gene expressions described above. By regulating in the transcriptional level of nitrogen assimilatory genes, the synthesis of free amino acids, proteins, chlorophyll and the availability of carbon and nitrogen resources are tightly controlled.

The effects of PII overexpressing and PII truncated overexpressing lines seemed to be very complicated as shown in the experimental data. With reference to the PII model in bacteria, PII is a functional trimer protein. Though it is unknown if PII form trimers in plants, it is highly likely to be a signaling protein with multiple subunits.

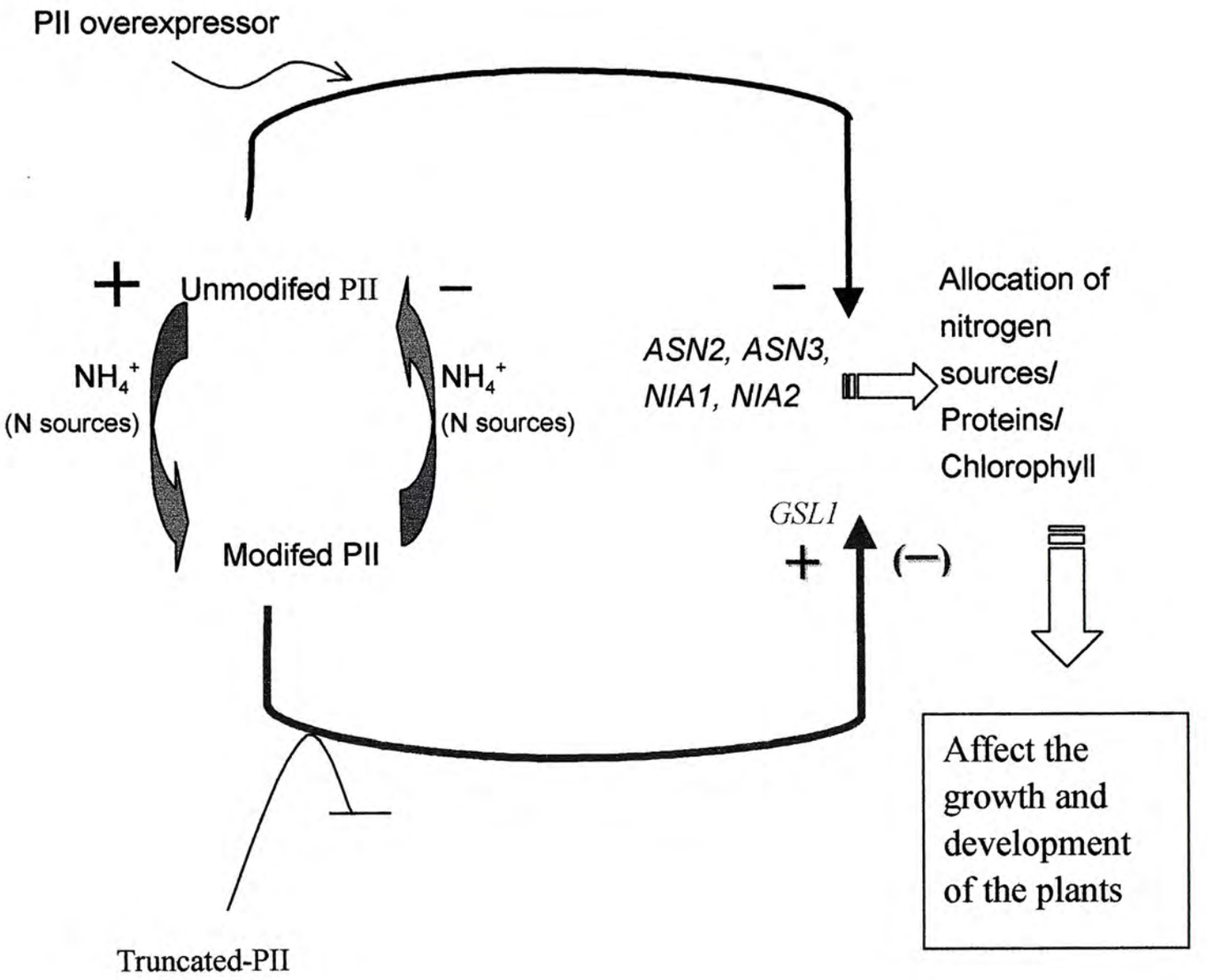


Figure 4.1: Proposed model for the roles of PII protein.

Overexpressing PII mRNA in PII overexpressing transgenic lines may produce excessive native PII subunits that may favor the accumulation of unmodified PII molecules.

In truncated PII overexpressing transgenic lines, the translated truncated PII subunits may bind to the native subunits and form non-functional or malfunctional PII proteins. It is possible that the presence of such aberrant PII protein will influence the action of the normal PII molecules.

4.9 Conclusions

Experimental data strongly suggested that gene expression patterns, chlorophyll contents under ammonium stress, nitrogen storage and root growth were all significantly altered in the PII overexpressing and truncated PII overexpressing transgenic lines. When grown in soil, it was undoubtedly that the truncated lines showed retarded growth and abnormal development. It may, according to the proposed model, due to the overexpression of truncated PII mRNA or native PII mRNA in the transgenic plants and thus disturb the normal signaling pathways regulated by PII.

4.10 Further studies

The phenotype of the two truncate lines, TPII6-9T2 and TPII3-6T2 seemed to be quite different in their phenotypes. Such discrepancies can be explained by several possible reasons. 1) Different steady-state levels of the truncated PII mRNA in the two lines were observed, especially when grown in soil. TPII3-6T2 has a higher expression level of truncated PII and it may then exert more severe effects on plants as observed in most experiments performed; 2) another possibility is due to the positional effect of the inserted DNA. There may have some unknown factors in addition to PII affecting plant growth. Therefore, more PII and truncated PII overexpressing transgenic lines are required for the analysis.

The PII transgenic plants, especially the truncated lines, showed alteration in the expression of *GSL1* and *ASN2* genes. In one of the truncated lines, TPII3-6T2, chlorosis was observed under high light intensities or when growth on MS agar plates. Since *ASN2* and *GSL1* genes have been suggested to be involved in photorespiration, further work may done to observe whether these transgenic plants have altered photorespiration rate and the to investigate whether PII protein is a crucial protein in signaling photorespiration.

According to the nitrogen-signaling pathway in bacteria, PII does not function in its

only existence. Instead, many other components are involved and all these components have not yet been found in plants. If PII remained to be a highly conserved regulatory protein across different realms of life, it is not surprise to find the corresponding components in plants. Therefore, further works should be focused on this aspect in order to delineate the roles of PII in nitrogen signaling pathway in plants.

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